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STUDIES IN NUTRITIONAL ANAEMIA

SOME DIETARY FACTORS INVOLVED IN HAEMOGLOBIN SYNTHESIS

by

REGINALD CLARENCE MacKENZIE HAMILTON
B.Sc. (Alberta), M.D. (Alberta).

Robert Tegler Research Scholar

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A thesis submitted from the Department of Biochemistry
in partial fulfilment of the requirements for
the degree of Master of Science at the
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INTRODUCTION

Nutritional anaemia, as the name implies, results directly from the lack of some dietary factor or factors necessary for haemoglobin formation, which may be either quantitative or qualitative. It is regarded as a secondary anaemia, as distinct from the primary, idiopathic or pernicious type. Clinically, secondary anaemia may be only one indication of what is actually a complex disease picture. The nutritional type, in common with many other secondaries, is characterized by a reduced amount of haemoglobin in the blood, a deficient number of erythrocytes, and a low colour index. In other secondaries, the true cause is more apparent, e.g., those which result from acute or chronic haemorrhage and those associated with certain specific diseases.

In anaemias generally, iron therapy has been in use for a long time. According to Sollmann (1920) the ancients treated anaemia by drinking water in which swords had been allowed to rust. Since Menghini in 1747 (quoted from Musser and Wintrobe, 1931) first detected the presence of iron in the blood this metal has been extensively used, and today enjoys great popularity among clinicians.

It is well known that during the last fifty years an enormous amount of work has been done on various aspects of iron metabolism, and that an extensive literature on the subject has been assembled. We have confined our review to the more important contributions, and especially to those which have a direct bearing upon our present knowledge of the dietary factors involved in haemoglobin synthesis.

The first, and most fundamental observations, were those made by Bunge (1890) after having completed painstaking research on the nature of food iron. He concluded that the iron in milk, eggs, certain plants, etc., was entirely in the form of a complicated organic compound. To quote from his lectures of 1890:-

> "In order to become acquainted with the precursors of haemoglobin I have examined the compounds of iron in the yolk of eggs and in milk. Yolk of egg contains no haemoglobin. It must, however, contain a precursor of the latter, for when the egg is incubated haemoglobin is formed from its constituents without extraneous aid."

He found no inorganic iron in egg yolk but succeeded in isolating from a peptic digest of the yolk protein, a substance which he found contained almost all of the iron of the yolk in an organic combination. This iron compound he named "haematogen" because of its resemblance, at least so far as empirical constitution was concerned, to haemoglobin. He believed the iron in all foods to be in this form, and as such absorbed and assimilated to produce haemoglobin. He believed that inorganic iron salts were not absorbed by the organism, a contention which he found difficult to reconcile with the fact that inorganic preparations of iron promote the formation of haemoglobin in chlorotic patients. He

has suggested, however, that the organic compounds of iron are in some way protected from decomposition in the digestive canal by preparations of iron. He had shown that ammonium sulphide gradually separated the iron from organic compounds of iron. Alkaline combinations of sulphur are found in the intestine, especially in the digestive disturbance associated with chlorosis. Therefore, if inorganic compounds of iron are present, they will at once fix the sulphur of the alkaline sulphides before it can act upon the organic compounds of iron. The latter are thus preserved from decomposition, and are absorbed.

About the same time, Abderhalden (1900) observed that if animals were kept for long periods on a whole milk diet, they became anaemic. Additions of inorganic iron to the milk did not cure the anaemia, although growth was promoted. This observation he considered did not preclude the possibility of inorganic iron participation in the synthesis of haemoglobin in normal nutrition, but indicated that milk lacked substances other than iron which were necessary for haemoglobin formation.

One year later, Bunge (1901) showed that young animals are born with an extra store of iron in the spleen and liver, and that these reserves become sources of iron for haemoglobin synthesis during the early period of the animal's life when they subsist wholly upon milk. He had previously shown (1884) that milk is poor in iron.

Fifteen years later, it became necessary, as the result of researches conducted in the intervening years, to modify Bunge's

views, because it was found that inorganic iron salts, as such, were utilized. Inorganic iron salts, however, were found not to be nutritionally equivalent to foods rich in organic iron compounds such as occur in green vegetables and egg yolk.

Attempts were then made to explain this finding, not by attributing any particular virtue to organic iron but on the grounds that the organism was dependent on its food for certain organic radicles related to haematin which were required for haemoglobin formation.

Quoting Robertson (1920) "There is every reason for supposing that the pyrrole group cannot be synthesized by the animals but must be obtained by them preformed; that is to say, from tissues of plants or from the tissues of animals which acquired it from plants".

Green vegetables, egg yolk, etc., were thus to be considered good foods for haemoglobin production, not because of the nature of the iron but because they were also good sources of the pyrrole nucleus. These observations were no more than a confirmation of Abderhalden's original contention. Hart and his colleagues (1925) further showed that rabbits made anaemic by subsistence on a whole milk diet, were not cured by adding inorganic iron alone, but were cured when iron-free extracts of cabbage or iron-free chlorophyll were fed in addition to the inorganic iron.

In later work by Hart, Steenbock,Elvehjem,Waddell,Herrin,

(1927,1928[3],1929), this supplementary effect of iron-free cabbage extract and of chlorophyll was duplicated by adding to the milk, inorganic iron and the ashes of these materials. Further experiments led them to conclude that copper was an essential supplement to inorganic iron in promoting haemoglobin regeneration in nutritional (milk) anaemia. Their results were clear cut and the evidence extremely convincing. It is the more surprising then that this discovery should have given rise to so much controversy.

Most of the contention centres around the specificity of copper when fed with iron in bringing about haemoglobin regeneration in nutritional anaemia. The following have come to the same conclusion as Hart and his colleagues:- McHargue, Healy, and Hill (1928); Titus, Cave, and Hughes (1928), McCollum and Becker (1930); Lewis, Weichselbaum, and McGhee (1930); Krauss (1930); Underhill, Orten,and Lewis (1931); Keil and Nelson (1931-B), and Cunningham (1931). Other investigators - Beard and Myers (1929-2), Drabkin and Waggoner (1930), Keil and Nelson (1931-A), have obtained recovery from "purified" inorganic iron alone. Titus, Cave, and Hughes found that manganese effectively supplemented iron, although since that time the ineffectiveness of manganese with iron, has been shown by several workers. (Waddell et al (1929); Weichselbaum (1930); Underhill, Orten, and Lewis). Beard and Myers (1929,1931) report other metals to be as good supplements as copper, while Mitchell and Miller (1929), contend that a complex of "trace"

elements is necessary. Waddell and his colleagues report no effect with those twelve trace elements. Drabkin and Miller (1931) have claimed that the anaemia produced by milk feeding was due not only to an iron deficiency but also to a deficiency of the pyrrole nuclei necessary in the formation of the porphyrin moiety of haemoglobin. To support this claim they showed that they could relieve the anaemia by feeding iron along with certain amino acids, such as arginine and glutamic acid. Elvehjem, Steenbock and Hart (1931), feeding glutamic acid, reported by Drabkin and Miller to be effective, have found their preparation of the same acid to be completely inactive.

The nutritional (milk) anaemia is produced by a lack of iron in the milk, so that assuming the presence of copper to be necessary for the utilization of inorganic iron, the regeneration of haemoglobin by inorganic iron alone will depend on whether or not the milk already contains sufficient copper. It must be remembered that it is necessary to add only a very small amount of copper (0.01 mg. per rat per day) to a relatively considerable amount of copper (0.15 mg. per litre according to Elvehjem and Lindow, 1929) already present in the milk in order to promote haemoglobin formation. Recently McFarlane (1932) has produced evidence to show that the methods previously in use for determining the copper content of cows' milk are inaccurate. It is probable that the above mentioned controversy would not have arisen had the various investigators had an accurate knowledge of the copper content of the milk they were feeding.

STATEMENT OF THE PROBLEM

It was planned to conduct the following investigations:-

1. To improve the experimental technique used in studying nutritional anaemia, particularly in regard to haemoglobin and copper determinations.

2. To confirm the essential role of copper in haemoglobin synthesis by conducting carefully controlled feeding experiments on nutritional anaemia (milk) in the rat, the copper content of the milk to be accurately determined.

3. To determine whether the addition of pure glutamic acid hydrochloride and iron to milk would promote haemoglobin regeneration.

4. To study the mechanism of the action of copper or glutamic acid hydrochloride, should one, or both of these substances be shown to stimulate, or be essential for haemoglobin synthesis.

(a) Determining the iron ("organic" and inorganic), and copper content of the liver of normal and of anaemic rats, and of rats in which the anaemia has been cured.

(b) Investigating the metabolism of iron ("organic" and inorganic) and copper during the embryonic development of the chick.

(c) Conducting feeding experiments with naturally occurring "organic" iron compounds such as lecitho-vitellin or haematogen in the absence of copper.

IMPROVEMENTS IN EXPERIMENTAL TECHNIQUE

Improvements in experimental technique in recent years have aided in bringing about a more careful study of nutritional anaemia. One illustration will suffice, namely, the method of developing experimental anaemias. In the main there are three satisfactory ways of bringing about this state. The first is haemorrhagic. This method has been used for many years and has been employed extensively by Whipple and Robscheit-Robbins during the last seven years. Neither this type of anaemia nor the therapy relative to it will be considered since it does not come within the scope of this paper. The next method is that of "milk with modifications". The milk is modified by adding either traces of minerals or by adding certain single salts as sodium citrate. By use of this method an anaemia in rabbits is readily secured. The last method is that of "milk alone". Since Waddell, Steenbock, Elvehjem, and Hart (1928) first demonstrated the suitability of the rat for studies in nutritional anaemia, this method has been generally used to develop anaemia in the first generation of rats. Quite recently, Elvehjem and Kemmerer (1931) have further modified this procedure so that young rats may be made anaemic in a few weeks.

Other recent improvements in experimental technique are:-

1. The purification of the dietary supplements used.

2. The development of more accurate methods of determining minute traces of Iron (Kennedy,(1930)) and Copper (McFarlane,(1932)).

3. The use of glass cages.

4. An accurate method of determining the amount of haemoglobin in minute samples of blood.

Our contribution to improvements in technique consisted of a modified method of determining copper (McFarlane) and a study of methods of determining haemoglobin in small samples of blood (McFarlane and Hamilton, (1932)).

Although there are many good clinical methods of determining haemoglobin there are none which we considered wholly satisfactory for a study of anaemia in the rat. The Newcomer method (1919,1923) has been used extensively by workers in this field. Wu (1923) described a method which he called "an ultra-micro-method for the determination of haemoglobin as a peroxidase" by which the haemoglobin content of 0.0001 c.c. of blood can be determined.

In so far as our requirements were concerned the method of Wu had a number of advantages over that of Newcomer:-

1. In the Newcomer method 0.02 c.c. of blood must be secured from the rat, while in the modified Wu method only 0.0025 c.c. is required to make an accurate estimation. The former involves severe snipping of the tail and in consequence an unnecessary loss of blood.

2. With anaemic rats "milking" of the tail is often necessary to obtain sufficient blood for the Newcomer method. This leads to inaccurate results.

3. It is often difficult to avoid rapid development of a cloudiness in the standard acid haematin solution. No such difficulty is encountered with Wu's method. Using the standard coloured glass disc is quite unsatisfactory with rat's blood.

4. The haemoglobin content of the blood of very anaemic rats (3-4 gms. Hb. per 100 c.c.) can be determined by the Newcomer method only when twice the usual quantity of blood (i.e. 0.04 c.c.) is used.

THE FACTORS WHICH AFFECT THE BLOOD-BENZIDINE REACTION AS APPLIED TO THE ESTIMATION OF HAEMOGLOBIN

The results of this investigation have been prepared for publication. McFarlane and Hamilton (1932). The type-written manuscript is hereto appended.

TECHNIQUE OF FEEDING EXPERIMENTS

As these feeding experiments must be conducted under the most carefully controlled conditions, it is important that the technique which we have adopted be described in detail.

CARE AND HOUSING OF EXPERIMENTAL ANIMALS

Both male and female rats were used in these experiments. They were the young of parents fed upon our stock ration, consisting of the following:-

1.	Cracked yellow corn	228 grams	BASAL MIXTURE
2.	Linseed oil meal	48 "	
3.	Crude Casein	15 "	
4.	Ground Alfalfa	6 "	
5.	Iodized Salt	1.5 "	
6.	Calcium Carbonate	1.5 "	

This was mixed, and to it were added 100 gms. Klim (powdered whole milk), i.e. three parts basal mixture to one part Klim.

The rats were born and reared in galvanized iron cages constructed locally according to the plans and specifications of Professor George Hunter. The young rats were all weaned at twenty-one days and removed to specially tinned galvanized iron cages,

with four or five rats in each group. In these cages they were fed only whole milk (cow's). Four to six weeks after weaning they were decidedly anaemic.

The milk used in these experiments was milked directly into cleaned (soap and water and distilled water) glass bottles. The bottle caps were of paper without metal clips.

The cover of a petri dish was used as a milk receptacle until the animals increased their milk consumption to the point where larger glass dishes were necessary. They were cleaned daily with soap and water, and rinsed with distilled water.

HAEMOGLOBIN DETERMINATIONS

Method of securing the blood sample: The rat was removed from its cage and its tail wiped clean from all adhering material. If the animal was very anaemic its tail was dipped in warm water and then dried. The tip of the tail was snipped off with a pair of sharp scissors and the first two drops of blood discarded. A part of the third drop of blood was pipetted to the .05 mark of a specially calibrated red blood cell pipette of the Thoma type. While an assistant immediately proceeded to cauterize the tail with a hot spatula to avoid unnecessary loss of blood, the operator wiped the tip of the pipette free of blood, and discharged the contents of the pipette into 3-4 c.c. of distilled water in a 10 c.c. volumetric flask. The pipette was flushed five times with the

solution, the liquid being drawn only as high as the 1.0 mark. The contents of the flask were then made up to the 10 c.c. mark with distilled water and thoroughly mixed. 1 c.c. of this solution was used for the determination.

Method of haemoglobin determination: The sensitive colorimetric method of Wu based upon the "pseudoperoxidase" action of haemoglobin was used. This blood-benzidine reaction has been investigated by McFarlane and Hamilton. We have adopted the following procedure in our routine haemoglobin estimation:-

1 c.c. of the diluted blood is pipetted into a 25 c.c. glass stoppered cylinder. 2 c.c. of a 2 per cent. benzidine solution in glacial acetic acid and 1 c.c. of 1.5 per cent hydrogen peroxide solution are added, allowed to stand one hour, then diluted with 21 c.c. of 25 per cent. acetic acid and mixed. At least ten minutes are allowed for full colour development to take place. The intensity of colour compared to that developed by the standard haemoglobin solution treated in this way was determined colorimetrically. The readings were made according to the recommendations of Hunter and Eagles (1925). Determinations were usually made every seven to ten days and at the same time the rats were weighed.

Preparation of the standard haemoglobin solution: The oxygen capacity of an oxalated sample of human blood was determined by the method of Sendroy (1931). 10 c.c. of this blood were diluted with 1 per cent. boric acid to give a solution con-

taining approximately 20 mgs. of haemoglobin per c.c. Preservation of the standard with 1 per cent. boric acid (Brown and Hill, 1923) is a decided improvement over the use of 4 per cent. sodium fluoride as recommended by Wu. This stock solution was kept in an ice-box and a portion removed only when determinations were being made. After the solution had been well mixed, a portion was removed and allowed to come to room temperature. 1 c.c. was pipetted into a 500 c.c. volumetric flask containing a little distilled water. The solution was then made up to 500 c.c. with distilled water and thoroughly mixed. 1 c.c. of this dilution was used in the above determination. This standard blood solution was used only on the day it was prepared. The stock solution, however, was employed for one month or longer.

PREPARATION AND METHOD OF ADMINISTERING THE MILK SUPPLEMENTS:

Copper solution: 0.63 gm. of C.P. Copper Sulphate ($Cu\ SO_4 . 5\ H_2O$) crystals were dissolved in distilled water, 5 drops 5 $N.H_2SO_4$. added, and the solution diluted to one litre.

This solution contained 0.1 mg. of copper per c.c. When copper was used as a supplement each rat received 0.25 c.c. (0.05 mg.) of this solution daily.

Iron solution: I. 7.27 gms. of C.P. Ferric Chloride ($FeCl_3 . 6\ H_2O$.) were dissolved in distilled water and diluted to one litre.

The solution contained 2.0 mgs. of iron per c.c. When iron was used as a supplement each rat received 0.25 c.c. (0.5 mg.) of this solution daily.

Iron solution II: (specially purified iron). The reagents used to purify the iron were all found, on testing, to be free from copper.

2 gms. of "analytical" iron wire were dissolved in a minimum amount of concentrated hydrochloric acid, and the iron oxidized to the ferric state by 2 c.c. of concentrated nitric acid. The solution was then evaporated to dryness on a sand bath and finally strongly heated to drive off the last traces of nitric acid. The residue was dissolved in concentrated hydrochloric acid and hydrogen sulphide bubbled into the hot solution contained in a glass stoppered flask. The flask was shaken and hydrogen sulphide again bubbled through the solution. Although no precipitate could be seen, the solution was twice filtered through specially cleaned Jena glass filters, then evaporated to dryness, placed in a desiccator and weighed as ferric chloride. The ferric chloride was dissolved in a very small amount of dilute hydrochloric acid and diluted with water. This solution was found on analysis to contain 0.168 gm. of iron per 100 c.c.

This solution contained 1.68 mgs. of iron per c.c. When iron was used as a supplement each rat received 0.25 c.c. (0.42 mg.) of this solution daily.

Glutamic acid hydrochloride (Sample I): Our glutamic acid hydrochloride was prepared from "practical" d-glutamic acid*

*Eastman Kodak Company

100 gms. of this glutamic acid were dissolved in one litre of boiling water. The solution was decolorized by boiling twice with animal charcoal, filtered, cooled, and then saturated with hydrochloric acid. (The hydrochloric acid was generated from NaCl and H_2SO_4.). The receiving beaker containing the glutamic acid was immersed in ice. When saturated, the solution in which the crystals had now begun to form was placed in the ice-box overnight. Next morning, the crystals were filtered on a Buchner funnel. The crystals were redissolved in a minimum amount of hot water, re-saturated with hydrochloric acid and placed in the ice-box over-night. The glutamic acid hydrochloride was thrice recrystallized. The crystals of glutamic acid hydrochloride were washed repeatedly with ice cold absolute alcohol and finally air dried.

Glutamic acid hydrochloride (Sample II): At a later date, a second sample of glutamic acid hydrochloride was prepared, particular precautions being taken to avoid copper contamination. In general the same procedure was again used in its preparation. Without using animal charcoal to decolorize, the glutamic acid was directly and repeatedly crystallized by saturating the solution with hydrochloride acid. The crystals which formed on each occasion were filtered upon specially cleaned Jena glass filters.* These crystals were washed each time with cold concentrated hydrochloric acid. The pure white crystals finally obtained were air dried.

*The Jena glass filters were cleaned by washing with hot cleaning solution, hot hydrochloric acid, nitric acid, and finally washed several times with water distilled from glass.

Both samples were examined for copper by the method of McFarlane. 2 gms. of sample I gave a positive test for copper with the sodium diethyldithiocarbamate reagent. The amount of copper was, however, too small to be determined. By this method of analysis 0.002 mg. of copper can be determined. Sample II analyzed by the same method contained no copper. 5 gms. of Sample I were analyzed by the method of Elvehjem and Hart (1931) and found to contain no copper.

7 gms. of glutamic acid hydrochloride were dissolved in 100 c.c. of distilled water. This solution contained 70 mgs. of glutamic acid hydrochloride per c.c. When the solution was used as a supplement each rat received 1 c.c. (70 mgs.) daily.

Method of adding milk supplements: The flasks containing the solution and the iron solution were kept in a refrigerator. These flasks were stoppered with perforated corks in which a pipette was fixed. This was done to avoid mixing the pipettes. Each morning these solutions, in the amounts already indicated, were added directly to the first small milk feeding.

INITIAL HAEMOGLOBIN LEVEL BEFORE ADDING MILK SUPPLEMENTS

Mitchell and Miller (1931) contend that to determine the extent to which a particular supplement stimulates haemoglobin regeneration it is essential that the degree of anaemia be as nearly as possible the same in all rats. Only by reducing the

haemoglobin content of the blood to a uniform level can the results of different investigators be compared. We are in complete accord with this viewpoint, and our practice has thus been to keep our animals upon a milk diet until the haemoglobin is reduced to 5 gms. per 100 c.c., or slightly less. Beard and Myers (1931) and Krauss (1931) have added their milk supplements when the haemoglobin content of the blood was reduced to 6 gms. per 100 c.c., or less. Other investigators allow much wider variations in the initial haemoglobin level.

PRELIMINARY FEEDING EXPERIMENTS

At the time the writer commenced his investigations there had been started in the laboratory some experiments on anaemia. Although these experiments were not being conducted under carefully controlled conditions he decided as a preliminary to carry them to completion. The results, were found to be of considerable interest.

The experiments were conducted as follows:-

Young rats, housed in tinned galvanized iron cages, were made anaemic in the manner already outlined. This state was indicated by the reduction in the milk consumption, sluggishness of movement, loss of sleekness of fur, and pallor of tail and ears. The rats were divided into three groups, each group being placed in a separate cage. The severity of the anaemia was not determined, but all the rats were made anaemic in one group and were the same age. Supplemental feedings were then commenced. The first haemoglobin determination was made one week after the start of supplementary feedings. The results are tabulated in Table I and shown graphically in Figure I.

In spite of the fact that the ferric chloride solution was not purified, the experiment gives a clear cut demonstration of the essential role of copper as a supplement to iron in haemoglobin synthesis.

A number of young rats were also fed (from weaning) on milk to which ~~were~~ was added 3 gms. of powdered egg-albumen to each 100 c.c. It was hoped that the extra protein would improve the milk con-

sumption which invariably decreases as the severity of the anaemia increases. The experiment was in all other respects a repetition of that just described. The results are tabulated in Table II and shown graphically in Figure II.

The toxic effect of feeding unheated egg albumen to rats, already observed by various workers, is confirmed in these experiments. It is of interest to observe that only in Group IV, with iron and copper added to the diet, was a fairly good response to haemoglobin production obtained. In spite of this improvement, the mortality was the same as in the other groups.

The diet of rats numbers 30 and 36 was further modified. The changes in their diet and the effects of such modifications are shown in Table III. For the sake of clarity the results are plotted in Figure III.

At the end of ten weeks on the milk-albumen diet, supplemented by iron, rat No.36 showed an increase in haemoglobin from 5.3 to 6.8 gms. per 100 c.c. Rat No.30, on the same diet, but without the iron supplement, showed a similar increase in haemoglobin from 3.4 to 5.30 gms. per 100 c.c. Upon adding to the diet of rat No.36 glutamic acid hydrochloride and eliminating the egg-albumen, a return to normal haemoglobin level occurred in five weeks. A similar response occurred with rat No.30. Thus the addition of iron to the milk-egg-albumen diet of an anaemic rat did not stimulate haemoglobin production when fed for ten weeks. The further supplementing of the iron-egg-albumen-milk diet with glutamic acid

hydrochloride cured the anaemia. The presence or absence of the egg-albumen did not change this result.

In view of the fact that the experiment involved only two rats, the results obtained cannot be accepted as conclusive.

FEEDING EXPERIMENTS

As in the preliminary experiments, both male and female rats were used, but another method of developing the anaemia, that recommended by Elvehjem and Kemmerer, has been used. From birth to weaning the young rats were allowed only the mother's milk and whole milk (cow's). The mother was removed from the cage once or twice daily and given solid food. She was carefully brushed free from adhering particles of food, etc., and returned to her young. The young were fed only whole milk (cow's) after weaning. By this method the anaemia is developed in six weeks after weaning and is of equal severity to that developed in eight to nine weeks by the previous method.

It was found in previous experiments that the milk, presumably the lactic acid, acted upon the specially tinned galvanized cages. We considered it advisable to eliminate this possible source of iron contamination, although rats housed in these cages, and fed upon milk only, do develop a severe anaemia. We constructed glass cages, according to the specifications of Underhill, Orten, and Lewis (1931). In these cages the possibility of any contamination of the rats' food is reduced to a minimum. It was found, unfortunately, that the newborn rats could not be raised satisfactorily in these glass cages. We were forced, therefore, to resort to the tinned galvanized iron cages until the young were weaned. They were then transferred to the glass cages.

The feeding dishes were specially cleaned and finally rinsed

in water distilled from glass. The same dish was always replaced in the same cage. The milk was collected directly into specially cleaned bottles, which were also finally rinsed several times with water distilled from glass. The source of the milk was not changed during the three months of the experiment. A sample of each month's milk supply was analysed for copper.

MONTH	COPPER CONTENT OF MILK IN MGS. PER LITRE
December	0.088
January	0.058
February	0.077

In these experiments iron solution No.II and glutamic acid hydrochloride sample II were used. The latter was dissolved in water distilled from glass.

In all other respects the experiment was carried out in the same manner as the preliminary feeding experiment.

When the rats from four litters were approximately six to seven weeks old, the development of an anaemia was evidenced by a reduced haemoglobin content of the blood, matting of the coat, general pallor, sluggishness of movement and a reduction in the milk consumption. When seven to eight weeks old the average haemoglobin content of the blood was 2-5 gms. per 100 c.c. The rats were kept another week on the milk diet to ensure a profound anaemia.

PLAN OF EXPERIMENT I

The anaemic rats were divided into four groups, each group

including some rats from each litter. At the conclusion of the experiment all the rats were to be killed. The iron, copper and glutathione content of the liver of each rat was to be determined.

The plan of the experiment , including additional information as to the condition of the rats (initial haemoglobin level), and the supplemental feedings are shown in Table IV.

RESULTS

In order to facilitate examination, the results, including the haemoglobin response (Figures IV, V and VI), and the growth response (Figures VII, VIII and IX), are condensed in tabular form in Table V.

DISCUSSION OF THE RESULTS

It may be concluded that the addition of copper and iron to the milk resulted in the most rapid cure of the anaemia, gave the best increase in growth and increased the food consumption by the largest amount. Taking into account the fact that Group III received only glutamic acid hydrochloride and no iron until the fifth day, no significant advantage accompanied the addition of glutamic acid hydrochloride to iron. Iron alone brought about a complete recovery from the anaemia although the rate of recovery was much slower than that obtained on further supplementing with copper. The effect of copper on the rate of growth, probably by stimulating the appetite, is noteworthy. It must be remembered, however, that those rats which received iron and copper had initially a slightly higher haemoglobin content of the blood than

those in the other groups. While we do not believe that this had any significant effect upon the final results, it must be considered an experimental error. Again the results with Group III must have been modified by the omission of the iron for the first five days.

From the above results it was also considered probable that if the amount of iron fed (0.5 mg. per rat per day) were reduced to 0.25 mg. per rat per day, the supplemental effect of copper and possibly also of glutamic acid hydrochloride might be still more pronounced. It was, therefore, decided to repeat the above experiment with the exception that 0.25 mg. of iron were fed per rat per day and care taken to eliminate the above mentioned errors.

PLAN OF EXPERIMENT II

The plan of the experiment, including additional information as to the condition of the rats (initial haemoglobin level), and the supplementary feedings are shown in Table VI.

RESULTS

Again to facilitate examination, the results, including haemoglobin response (Figure X),and the growth response (Figure XI), are condensed in tabular form, in Table VII.

DISCUSSION OF RESULTS

These results are much the same as those in the previous experiment. The supplementary effect of copper is, however, more

marked. Haemoglobin regeneration is remarkably rapid during the first ten days, when the milk diet is supplemented with iron and copper. The effect of iron alone was much the same as that with iron plus glutamic acid hydrochloride. The addition of glutamic acid hydrochloride was, however, accompanied by a definite though slight improvement.

It may be concluded from these results that the addition to the milk diet of pure iron results in a definite increase in haemoglobin. While the anaemia is ultimately cured by adding iron alone the recovery is, however, much slower than when the iron is supplemented with copper. This stimulatory effect of copper is not duplicated when an equal amount of iron is supplemented with glutamic acid hydrochloride. Again it would appear that the response elicited by copper depends upon the amount of iron being fed. It is surprising that the essential role of copper, so clearly demonstrated in the preliminary feeding experiment, is not shown in these carefully controlled experiments. We naturally expected that the indispensability of copper for haemoglobin formation would have been even more clearly demonstrated. We have no satisfactory explanation to offer for this conflicting result.

THE MECHANISM OF THE ACTION OF COPPER IN PROMOTING HAEMOGLOBIN BUILDING

The effect of copper in promoting the utilization of inorganic iron in haemoglobin synthesis has been confirmed and it is of importance to consider the mechanism of the reaction. The results of the feeding experiments suggest that copper feeding facilitates utilization of inorganic iron.

Cunningham (1931) has claimed that "copper feeding definitely increases the proportion of "organic" to total iron in the liver, and that this change from inorganic to "organic" iron is probably a step in haemoglobin building, depending on the presence of copper. If the function of copper in metabolism is confined to rendering inorganic iron available for haemoglobin formation, then organic food iron in the form of lecitho-vitellin or haematogen should promote haemoglobin synthesis without the presence of copper.

To determine whether this assumption was tenable, a considerable quantity (over 100 gms.) of lecitho-vitellin was prepared, which was to be fed as a supplement to the milk diet of anaemic rats, provided this protein contained no copper.

PREPARATION OF LECITHO-VITELLIN FROM EGG YOLK

In endeavouring to improve upon existing methods of preparing the pure protein we have benefited considerably from the

observations made by Kay and Marshall (1928) in the course of their preparation of livetin, the second protein of egg yolk.

The intact yolks were freed from egg white by washing first in running water and then under a slow stream of 0.9% NaCl on filter paper. The vitellin membrane was punctured so that the yolk contents were transferred to a beaker without the membrane and chalazae. The yolks diluted with an equal volume of 10 per cent. NaCl were repeatedly extracted with ether until the ether extract was colorless. This was carried out, using a mechanical shaker, in a refrigerator room at 15° F. and required five twelve-hour extractions, each with an amount of ether equal to the volume of the egg yolk solution. The saline solution was dialyzed in a large parchment dialyzer for two days at about 30° F. against repeated changes of distilled water. A large precipitate of the globulin forms. To complete the precipitation the solution was diluted with four volumes of ice cold water and after standing overnight was rapidly centrifuged. The protein was dissolved in a minimum amount of 10 per cent. NaCl, transferred to the dialyzer, and the precipitation and separation repeated. A third solution and precipitation was carried out with the loss of a small amount of the protein which failed to dissolve in 10 per cent. NaCl. The protein precipitate was transferred to an extraction bottle and extracted with alcohol-ether (seven parts alcohol and three parts ether) at 15° F. The extraction was repeated until no visible residue was obtained when 50 c.c.

of the extract were evaporated to dryness. The lecitho-vitellin was next extracted for twenty-four hours with anhydrous ether in a large glass Soxhlet apparatus with metallic sodium in the extraction flask. (Hewitt (1927)). The lecitho-vitellin was ground to a fine white powder and dried in a vacuum desiccator over sulphuric acid.

LECITHO-VITELLIN AS AN IRON AND COPPER CONTAINING PROTEIN

The sample of lecitho-vitellin was examined for copper by the method of McFarlane and was found to contain a surprisingly large amount compared to the quantities of copper generally found in biological materials. It happened that there was available at this time another sample of lecitho-vitellin. The two samples were analysed by Mr. W. D. McFarlane with the following results:-

Sample of lecitho-vitellin	% Copper	% Iron	Ratio Cu: Fe
1	0.0034	0.047	1 : 13.8
2	0.0032	0.045	1 : 13.4

NOTE: After decomposition of the organic substance the iron was determined by potentiometric titration with titanous sulphate (See McFarlane (1932.1)).

Egg yolk, according to McFarlane, Fulmer and Jukes (1930), contains approximately 2.5 gm. of protein and about 0.08 mg. Copper (average value from their Table IX). They found, however,that by changing the protein in the diet of the hen the total protein content of the egg yolk could be varied as much as 30 per cent. The copper content of the entire egg was also found to be an extremely variable quantity. Livetin con-

stitutes about one-fifth of the total yolk protein, according to Kay and Marshall (1928) and the total amount appears to be relatively constant from one egg to another. Assuming these average values represent approximately the total protein, livetin, and copper content of egg yolk; that the lecitho-vitellin contains all the copper; and also presupposing that the yolk contains only these two proteins, we find -

Copper content of the yolk	0.08 mg.
Total protein (vitellin - livetin)	2.5 g.
Livetin	0.5 g.
Vitellin content of yolk	2.0 g.

The copper content of lecitho-vitellin should be approximately -

$$\frac{0.08}{2.0} \times 100 = 4.0 \text{ mgs. per cent.}$$

Loeschke (1931) has obtained more constant values for the copper content of the hen's egg (0.055 - 0.095 mgs.) than those recorded by McFarlane et al just quoted. The copper content of lecitho-vitellin using the average of Loeschke's values should be about:-

$$\frac{0.066}{2.0} \times 100 = 3.3 \text{ mgs. per cent.}$$

These calculated values are probably only rough approximations. Since they are, however, of the same order of magnitude as the actual copper content of lecitho-vitellin it is not improbable that all the copper of the yolk is contained in this protein.

We still hoped that the iron containing prosthetic group of lecitho-vitellin could be prepared free from copper. Mr. McFarlane has found that when lecitho-vitellin is hydrolyzed with 0.25 N. NaOH at 37° C. for seventy-two hours, the iron and copper in this digest has been quantitatively split off from the protein molecule in a non-protein (not precipitated by trichloracetic acid) form. A substance or substances can be precipitated from the trichloracetic acid filtrate by neutral lead acetate. After decomposing this lead salt (or salts) with hydrogen sulphide the filtrate from the lead sulphide precipitation contains in organic combination all of the iron and about 25 per cent. of the copper of the lecitho-vitellin. Whether the presence of copper in the filtrate from the lead acetate precipitate was due to incomplete precipitation or to the presence of a second copper containing fraction in lecitho-vitellin was not determined.

In view of these observations, it was useless for us to continue with our original intentions so far as the feeding experiments were concerned. It is obvious, however, that the above findings are of unique significance. It is suggested by these results that in the lecitho-vitellin molecule there is a component containing iron and copper in organic combination, which possibly represents, at least in the chick, an intermediate stage in the formation of haemoglobin.

These observations indicate that one of the intermediate compounds formed in the synthesis of haemoglobin contains both iron and copper in firm organic combination. If such is the case these results prove conclusively the essential role of copper in haemoglobin synthesis.

THE EFFECT OF DIET AND AGE ON THE COPPER CONTENT OF RAT LIVERS

McHargue (1925), has observed that the newborn animal is well provided with copper and that the liver of the newborn rat has a higher copper content than the adult rat liver. He believes that there is a mobilization of copper just as there is of iron, in the foetus during pregnancy and suggests that this metal is stored in the liver along with iron to carry the animal over the critical stage in early life. These findings have been confirmed by Lindow, Peterson and Steenbock (1929) and by Cunningham (1931) for the rat, and by Morrison and Nash (1930) for the infant. Cunningham reports that the newborn rat liver contains more copper than the foetal. Lindow, Peterson and Steenbock (1929) noted that the rat liver in copper feeding shows the most striking increase of all the tissues of the body. There are great variations in the copper content of the infant liver and of the adult liver. (See Review of Morrison & Nash (1930)). Morrison and Nash show a wide variation in their own results of analysis of infant livers and found a very low copper content in the liver of an infant which had a severe anaemia.

The liver appears to reflect variations in the copper content of the diet, as well as in that of the body. We have studied the copper content of this organ in the normal rat, in the rat developing and recovering from nutritional anaemia, by applying the sodium diethyldithiocarbamate method of determining

copper (McFarlane (1932)).

METHOD OF REMOVING THE LIVER: The animal was killed by concussion. By means of a sharp pair of scissors a midline incision was made through the skin, and the belly muscles exposed by skinning as high as the upper part of the thorax and as low as the pelvis. This skin was cut away. The peritoneal cavity was opened by a midline incision from ziphoid process to the pubic symphysis and the peritoneum and muscles cut in such a way as to fold the wall laterally. The inferior surface of the liver was exposed by drawing the stomach to the left and the presenting intestines downward. The two small infero-posterior lobes of the rat liver were then freed of omental restraint by the use of scissors. The oesophagus was seized in a pair of forceps, severed and the stomach pulled well out of the field of operation. The liver was drawn down and its mesentery above cut as far back as the hepatic vein and vena cava. The junction of these two vessels was seized and severed just below the diaphragm. The liver was withdrawn, as the diaphragm was clipped from its upper surface by a pair of curved blunt scissors. The liver was then completely withdrawn, immediately freed from adhering blood and weighed on a tared watch glass or in an evaporating dish.*

The liver was dried in an electric oven at 100° C. to constant weight. The sample, in an evaporating dish, was first slowly charred over a low flame, then transferred to a muffle furnace (400° C.) and ashed in twelve hours. After cooling 2 c.c.

* The porcelain evaporating dishes were freed from copper by following the procedure recommended by Elvehjem and Lindow (1924)

of concentrated nitric acid was added, and the solution carefully evaporated to dryness. Spattering was prevented by stirring with a short glass rod. The ashing was completed by again heating for one hour in the muffle furnace. A perfectly white ash should be obtained. The oxidation with nitric acid was repeated if a white ash was not at first obtained.

After allowing the ash to cool 5 c.c. of 1-1 hydrochloric acid (18. gm. HCl per 100 c.c.) was added and the ash completely dissolved with the aid of heat and a glass stirring rod. The hydrochloric acid solution of the ash was carefully transferred to a 25 c.c. glass stoppered cylinder using 1 c.c. portions of distilled water to rinse the evaporating dish.

The remainder of the determination was carried out in essentially the same manner as outlined by McFarlane for the estimation of copper in blood with the exception that 3 c.c. of a saturated solution of sodium pyrophosphate and 3 c.c. of amyl alcohol were used.*

The tabulated results, in Table VIII, illustrate the accuracy with which the copper content of rat's liver can be determined. It has been further found that accurate copper determinations can be made on as little as 0.25 gms. of liver.

It may be concluded for these results (Table IX) that the rat is born with a large reserve of copper in the liver. This store of copper is slowly depleted during the development

* All reagents used were tested and found to be free from copper. Only water redistilled from glass was used.

of nutritional (milk) anaemia. In the anaemic rat (47 days old) the amount of copper per unit of dry liver is approximately one-tenth of the amount present in the newborn rat liver. These results confirm the findings of other workers cited above. The copper content of individual rat livers (newborn to 47 days old) are recorded for the first time. This was made possible by the micro nature of the method of analysis.

THE COPPER CONTENT OF THE CHICK LIVER DURING EMBRYONIC DEVELOPMENT:

All of the iron and copper necessary for the synthesis of haemoglobin in the blood of the embryo chick must obviously be present in the hen's egg. This closed system offers an excellent opportunity to study the factors effecting haemoglobin formation. Since part of the iron and copper in egg yolk is present in organic combination, it is of interest to determine whether the increase in the iron content of the liver during embryonic development of the chick, is paralleled by a similar increase in the copper content.

Loeschke (1931) has reported that the liver of the embryo chick is greatly enriched with copper during its development. In his experiments the chick was killed one to two days before hatching. The copper content of the liver was determined and the amount of copper compared with that in the rest of the body. Composites of three to five livers were required for analysis. The data recorded by him were very incomplete.

We have determined by the same method of analysis as described above -

1. The copper content of the livers of individual chick embryos at different stages of development.

2. The copper content of the chick's liver at hatching.

3. The copper content of the livers of three-day old chicks (fed only water).

The chick liver was excised in essentially the same manner as described for the removal of the rat liver. In the former, however, even greater care must be taken, particularly in separating the large gallbladder. The gallbladder contains copper so that if this organ is ruptured the liver sample must be discarded.

One difficulty which we have encountered in analyzing the chick liver should be mentioned. In the preliminary charring of the sample before ashing, there is a great tendency for the material to creep up the side of the evaporating dish. We have only been able to prevent this by charring at a very low temperature and at times applying the flame to the edge of the dish.

The results (Table X), although limited in number, indicate that the copper content of the embryonic liver increases during development. From the sixteenth to the nineteenth day of incubation there was practically no increase in the total copper content of the liver. The amount of copper per unit of dry liver markedly decreases as the embryo develops. This value for the hatching chick was only about one-fifth of that for the thirteen-day embryo. The total amount of copper in the liver of the hatched chick and the copper content per unit of dry liver remains practically unchanged after three days without food.

POSTCRIPT

The investigations recorded in this paper are obviously incomplete. Several aspects of nutritional anaemia outlined in the "Statement of the Problem" still remain to be studied. The interpretation of some of our results depends entirely on information which is still to be secured. It is obvious that the iron as well as the copper content of the liver must be known in order that a true picture of the mechanism of the action of copper may be obtained.

When determining the copper content of rat and chick livers, wherever possible a portion of each of the dried liver preparations was set aside for the estimation of "organic" and inorganic iron. The full interpretation of the results of the copper analysis must await this information. The data on the metabolism of copper during embryonic development is quite inadequate. Further experiments in this regard are being carried on. It is hoped that when the additional information is available, some light may be thrown on the part played by copper and dietary "organic" iron compounds in the synthesis of haemoglobin.

In reviewing the literature we have refrained from discussing many other important contributions to this subject which we have not actually touched but which might fittingly have been included.

TABLES AND GRAPHS

TABLE I

Group Number	Number of Rats	Rat Numbers	Supplements added to diet of anaemic rats	Hb determinations Time after supplements added 7 days	21 days
I	4	1	Continued on	5.1	2.66
		2	milk only	5.1	-
		5		-	3.99
		3		4.9	-
II	4	8	0.5 mg. Fe	10.1	8.0
		7	(as $FeCl_3$)	10.6	4.2
		10	per rat	10.7	-
		9	per day	8.7	6.1
III	3	11	0.5 mg. Fe	12.0	15.7
		12	(as $FeCl_3$)	10.9	13.9
		13	and 0.05 mg. Cu (as $CuSO_4$) per rat per day	12.4	15.4

TABLE II

Group Number	Number of Rats used	Rat Numbers	Supplements added to diet of anaemic rats	Haemoglobin determinations Time in weeks (after supp. added) 1	3	4	5	6	8	9	10	12
IV	5	31	Nil	7.5	-	*						
		32		7.7	-	*						
		33		-	*							
		34		-	-	*						
		30		10.8	3.4	-	-	-	-	-	5.3 **	-
V	5	35		12.6	-	-	*					
		37	0.5 mg. Fe (as $FeCl_3$) per rat per day	8.5	-	-	-	*				
		38		10.4	4.6	-	-	-	-	*		
		39		9.8	3.8	-	-	-	*			
		36		8.9	5.3	-	-	-	-	-	6.8 **	
VI	4	42	0.5 mg. Fe (as $FeCl_3$) and 0.05 mg.Cu (as $CuSO_4$) per rat per day	11.1	12.9	-	-	-	-	-12.9		*
		41		10.5	8.0	-	-	-	-	*		
		43		10.8	12.9	-	-	-	*			
		40		10.5	-	-	*					

* Died

** Diet further modified after this date

TABLE III

Rat Number	Haemoglobin Deter. (Rats on albumen-milk diet) Time in weeks 1	3	10	Supp. added to albumen milk diet	Changes in Supp. after 10 weeks	Haemoglobin Deter. Time in weeks (after supp. added) 2	5	9
36	8.9	5.3	6.8	0.5 mg. Fe (as $FeCl_3$)	Egg albumen discontinued 70 mgs. glutamic acid HCl substituted	10.0	14.7	11.9
30	10.8	3.4	5.3	Nil	70 mgs. glutamic acid HCl and 0.5 mg. Fe (as $FeCl_3$)	4.2	13.3	12.3

Note: The egg-albumen and glutamic acid HCl were by analysis copper-free.

TABLE IV

Group Number	Number of Rats	Rat Numbers	Initial Hb Level	Supplement added
I	8	2	4.4.	0.5 mg. Fe (as $FeCl_3$) and 0.05 mg. Cu (as $CuSO_4$)
		3	4.5	
		7	2.4	
		11	4.1	
		21	4.3	
		4	2.3	
		5	2.2	
		23	5.4	
II	6	1	2.0	0.5 mg. Fe (as $FeCl_3$)
		6	1.2	
		8	3.0	
		9	2.9	
		13	2.2	
		15	4.1	
III	5	14	2.3	0.5 mg. Fe (as $FeCl_3$) and 70 mgs. of glutamic acid hydrochloride
		16	3.3	
		17	3.7	
		18	3.1	
		20	2.6	
		22	4.3	
IV (control)	4	0	-	continued upon milk alone
		10		
		12		
		19		

Note: In Group I - Rats Nos.4,5 and 7, died during the first two days and were replaced by rats Nos.11,21 and 23. Rat No.2 was accidentally killed and No.3 died after 12 days.

In Group II - Rats 1 and 6 died after 12 days.

TABLE V

GRAMS OF HAEMOGLOBIN PER 100 C.C. OF BLOOD
(average of each group of rats)

Group Number	Initial	After 10 days	Increase	Initial	After 21 days	Increase
I	4.5	13.5	9.0	4.5	15.8	11.3
II	3.0	9.3	6.3	3.0	12.1	9.1
III	3.2	8.7	5.5	3.2	11.6	7.4

AVERAGE WEIGHT (GMS.) OF EACH GROUP OF RATS

Group Number	Initial	After 8 dys.	Inc. in 8 dys.	After 17 dys.	Inc.in 17 dys.	After 28 dys.	Inc.in 28 dys.
I	65	66	1	95	30	109	44
II	80	82	2	105	25	108	28
III	61	54	-7	73	12	82	21

FOOD CONSUMPTION (C.C. MILK PER RAT PER DAY)

Group Number	Initial	8th-d.	Inc.	17th-d.	Inc.	28th-d.	Inc.
I	20	36	16	65	45	66	46
II	20	30	10	51	31	50	30
III	20	27	7	33	13	50	30

TABLE VI

Group I	Number of Rats	Rat Numbers	Initial Hb Level	Supplement Added
A	3	1	3.3	0.25 mg. Fe (as $FeCl_3$) and 0.05 mg. Cu (as $CuSO_4$)
		2	4.7	
		3	6.9	
B	4	4	5.0	0.25 mg. Fe (as $FeCl_3$)
		5	2.0	
		6	7.6	
		7	3.4	
C	4	8	4.1	0.25 mg. Fe (as $FeCl_3$) and 70 mgs. Glutamic Acid HCl
		9	4.3	
		10	5.4	
		11	5.6	

Note: Rats 3,5,11, died 2 days and Rat No.9, 6 days after supplements were started.

TABLE VII

GRAMS OF HAEMOGLOBIN PER 100 C.C. OF BLOOD
(average of each group of rats)

Group Number	Initial	After 11 d.	Increase	Initial	After 24 d.	Increase
A	4.0	15.2	11.2	4.0	16.3	12.3
B	4.5	8.8	4.8	4.5	10.9	6.4
C	4.8	6.4	1.6	4.8	12.4	7.6

AVERAGE WEIGHT (GRAMS) OF EACH GROUP OF RATS

Group Number	Initial	After 11 d.	Increase in 11 d.	After 24 d.	Inc.in 24 d.	After 33 d.	Inc.in 33 d.
A	59	89	30	115	56	115	56
B	55	71	16	86	31	90	35
C	50	73	23	93	43	104	54

TABLE VIII

	Weight of liver taken for analysis (grams)	Mgs. of copper per gram of tissue	
		Fresh Weight of Liver	* Dry Weight of Liver
1. Normal adult rat (male)	1.6135	0.0059	0.0218
	1.3085	0.0056	0.0199
2. Normal adult rat (male)	1.4680	0.0062	0.0209
	1.8233	0.0057	0.0194
3. Normal adult rat (female)	1.2798	0.0069	0.0254
	1.0145	0.0074	0.0287
	0.9602	0.0071	0.0271

* The portion of liver tissue was dried at 100° C. for 12 hours.

TABLE IX

SHOWING THE EFFECT OF NUTRITIONAL (MILK) ANAEMIA, IN VARYING DEGREES OF SEVERITY, ON THE COPPER CONTENT OF RAT LIVER

Rat No.	No. of days on Anaemia Producing Diet of Elvehjem and Kemmerer	Copper Content of the Liver in Mgs. per Kg.	
		Fresh Weight	Dry Weight
1	Newborn -	24.6	120.8
5	" -	25.8	105.5
11	" -	28.5	115.5
8	" -	23.1	96.4
21	28	11.70	41.8
22	"	7.65	29.0
23	"	5.80	31.4
24	"	8.54	35.4
25	"	10.90	39.3
26	"	5.50	29.1
30	40	3.53	16.3
31	"	4.73	17.2
32	"	4.68	20.0
33	"	3.28	13.4
7	47	3.03	14.8
5	"	1.63	7.2
2	"	2.99	14.4
11	"	3.79	16.7
10	"	3.86	16.0
3	"	3.60	14.3
1	"	3.92	14.0
		4.32	16.9

TABLE X

Sample No.	Description of Sample Foetal Chick Approx. age in Days	Copper Content in Mgs. per Kg. of Liver		Total Copper in Liver Mgs.
		Fresh Weight	Dry Weight	
1	13	31.4	150.0	0.005
2	16	32.4	135.5	0.012
3	17	31.6	113.0	0.013
4	17	31.8	124.0	0.014
5	18	23.2	71.0	0.013
6	19	23.4	75.0	0.015
756	Hatching chick	12.5	31.6	0.010
768	" "	11.4	28.6	0.013
775	3 day old chick	11.2	28.1	0.010
770	" "	9.5	26.2	0.007
771	" "	12.4	32.4	0.013

Note: The gall bladder and its contents, taken from a hatching chick, contained 0.007 mg. of copper.

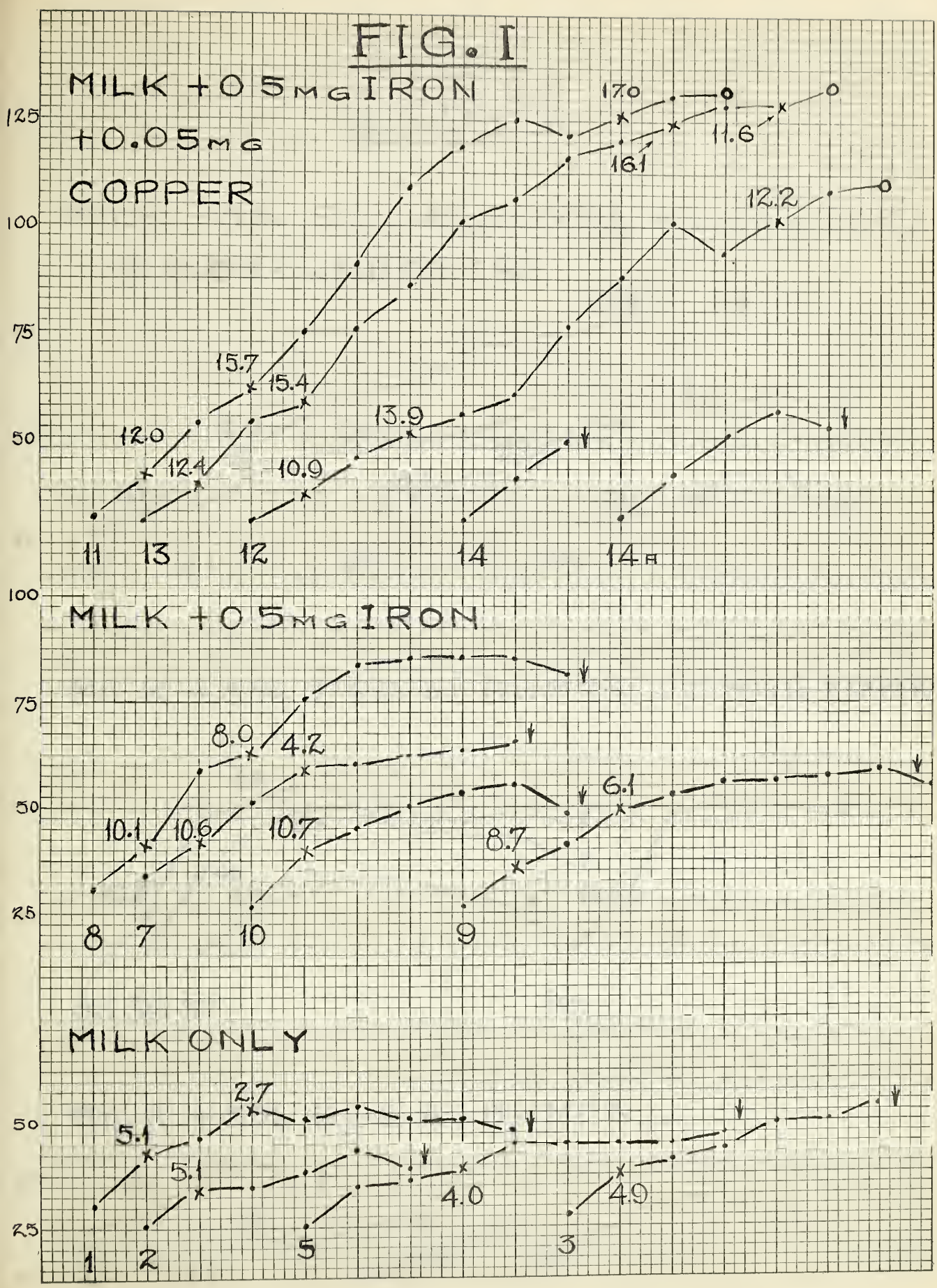
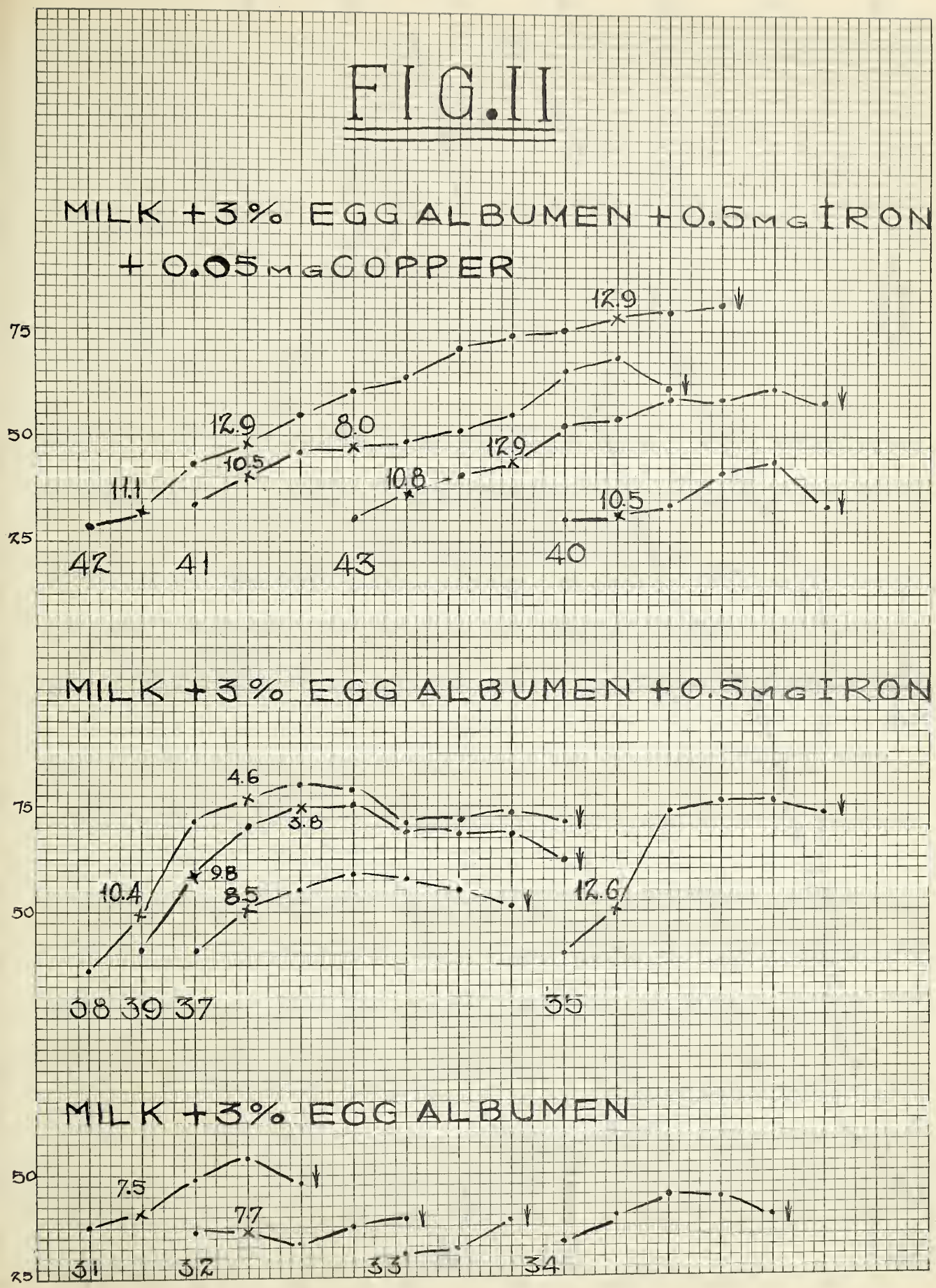
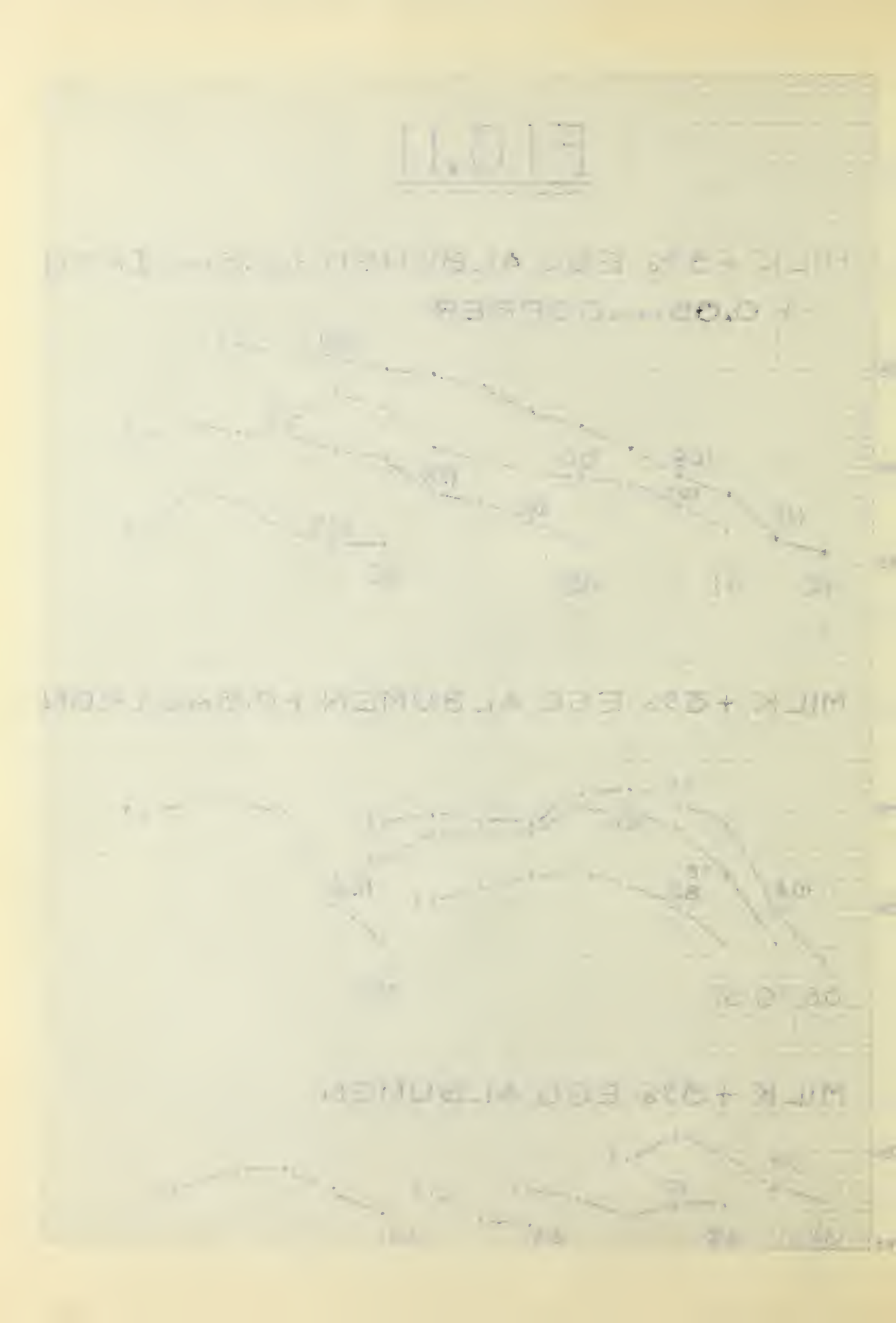


FIG. I
MILK +05MG IRON
+0.05MG
COPPER
125
100
75
50
17.0
11.6
16.1
12.2
15.7
15.4
12.0
12.4
13.9
10.9
11
13
12
14
14R
100
MILK +05MG IRON
75
50
25
8.0
4.2
6.1
10.1
10.6
10.7
8.7
8
7
10
9
MILK ONLY
50
25
2.7
5.1
5.1
4.0
4.9
1
2
5
3

FIG. II


MILK +3% EGG ALBUMEN +0.5mg IRON
+0.05mg COPPER
75
50
25
12.9
12.9
8.0
10.5
11.1
10.8
12.9
10.5
42
41
43
40
MILK +3% EGG ALBUMEN +0.5mg IRON
75
50
4.6
3.8
9.8
10.4
8.5
12.6
38
39
37
35
MILK +3% EGG ALBUMEN
50
25
7.5
7.7
31
32
33
34



GMS % HAEMOGLOBIN
WEIGHT IN GRAMS
16
14
12
10
8
6
4
200
175
150
125
100
75
50
Hb. 30
Hb. 36
WEIGHT
36
30
1st MODIFICATION STARTED
2nd MODIFICATION STARTED
ONE WEEK

FIG III

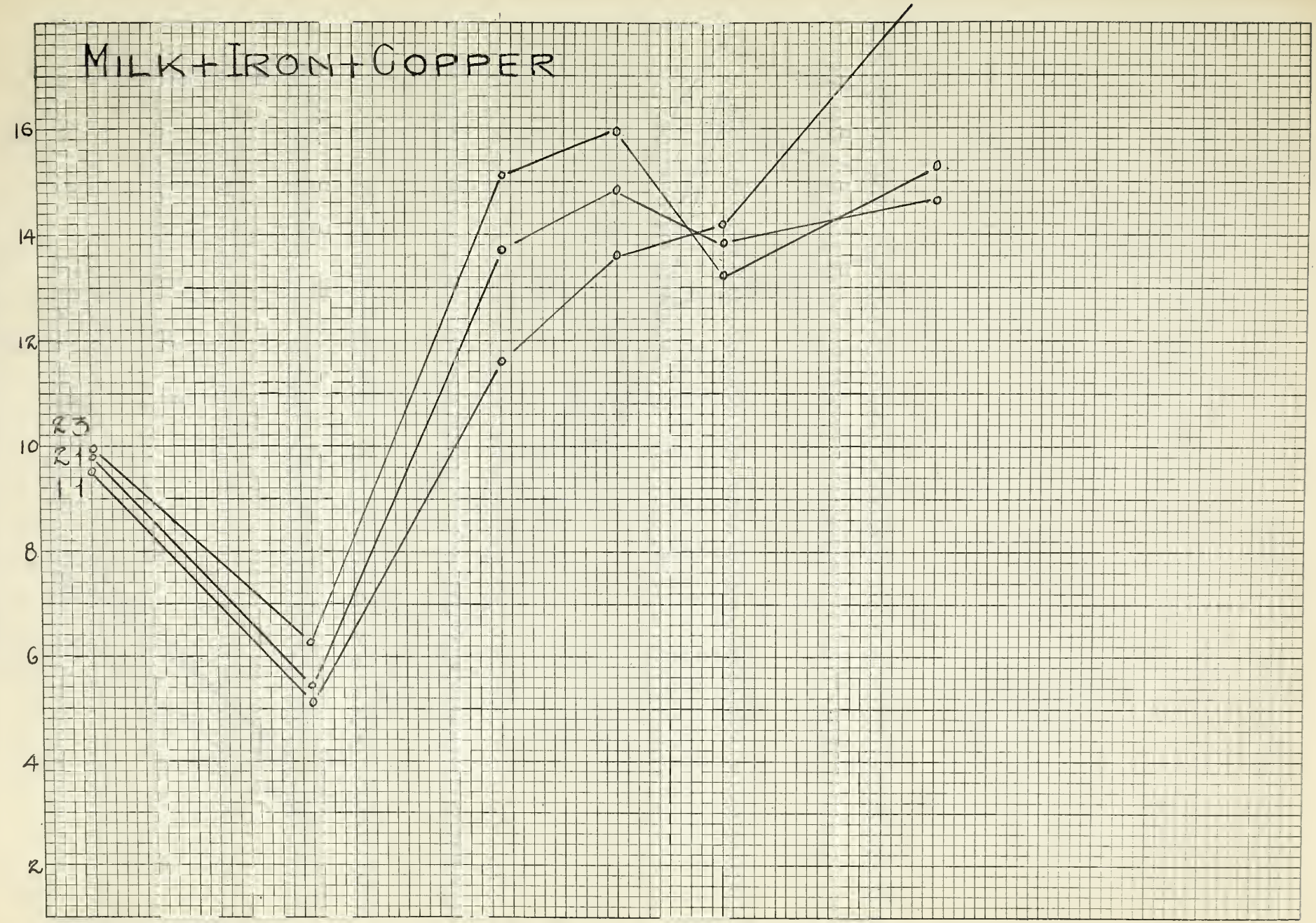

MILK + IRON + COPPER
GMS % HAEMOGLOBIN
16
14
12
10
8
6
4
2
23
21
11
TIME IN DAYS

FIG. IV

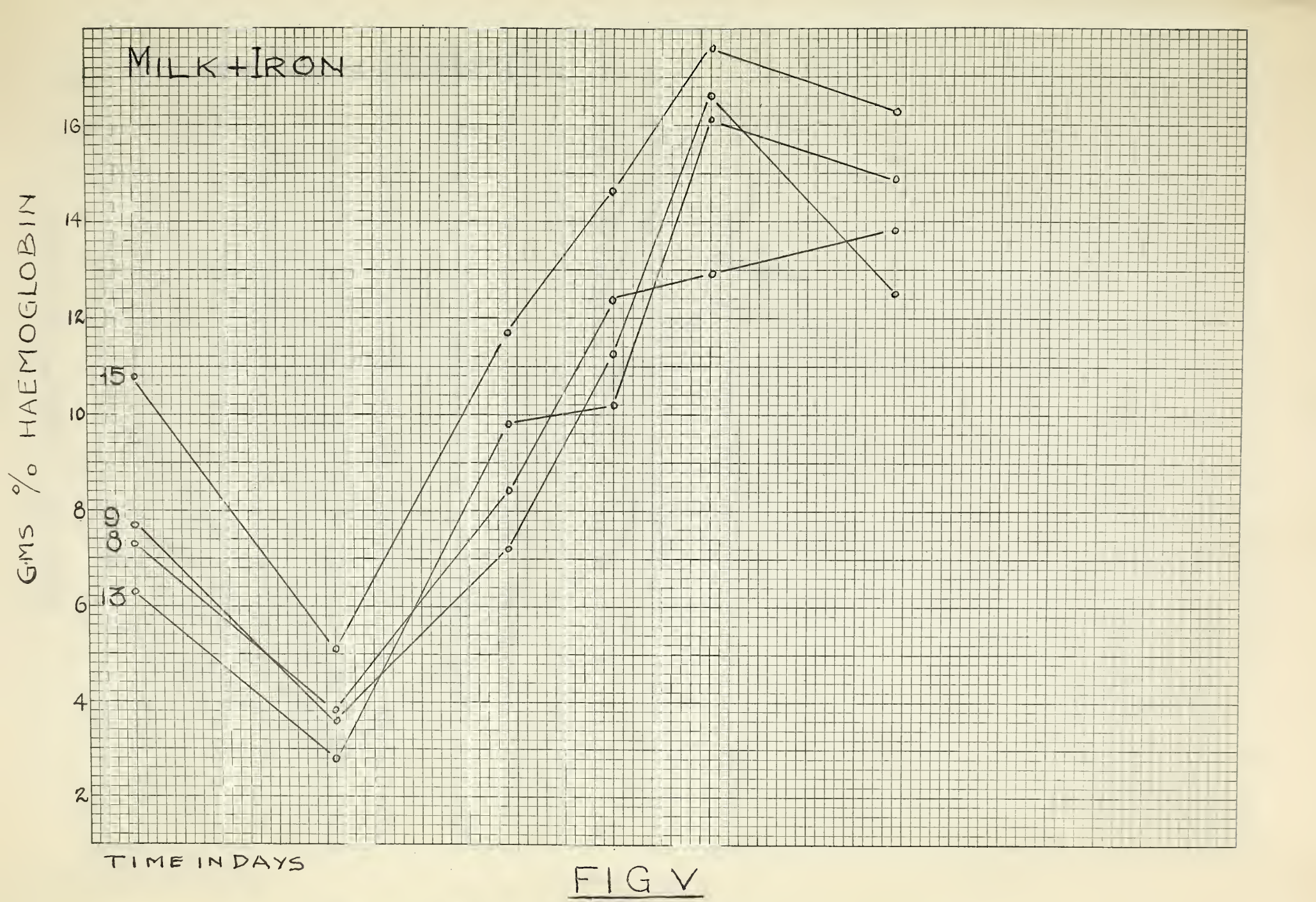


FIG V

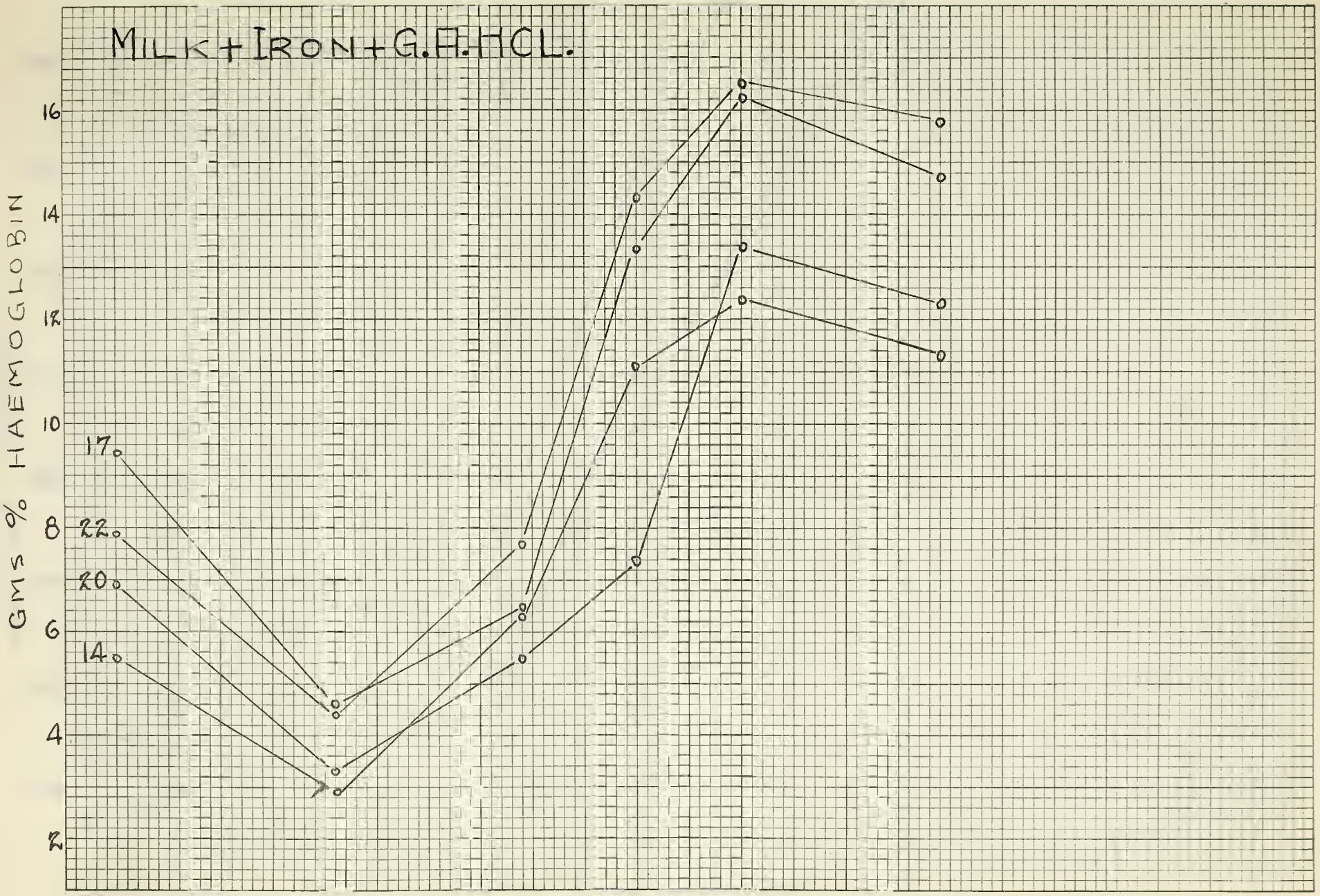

MILK + IRON + G.A. HCL.
GMS % HAEMOGLOBIN
16
14
12
10
8
6
4
2
17.
22.
20.
14.
TIME IN DAYS.

FIG VI

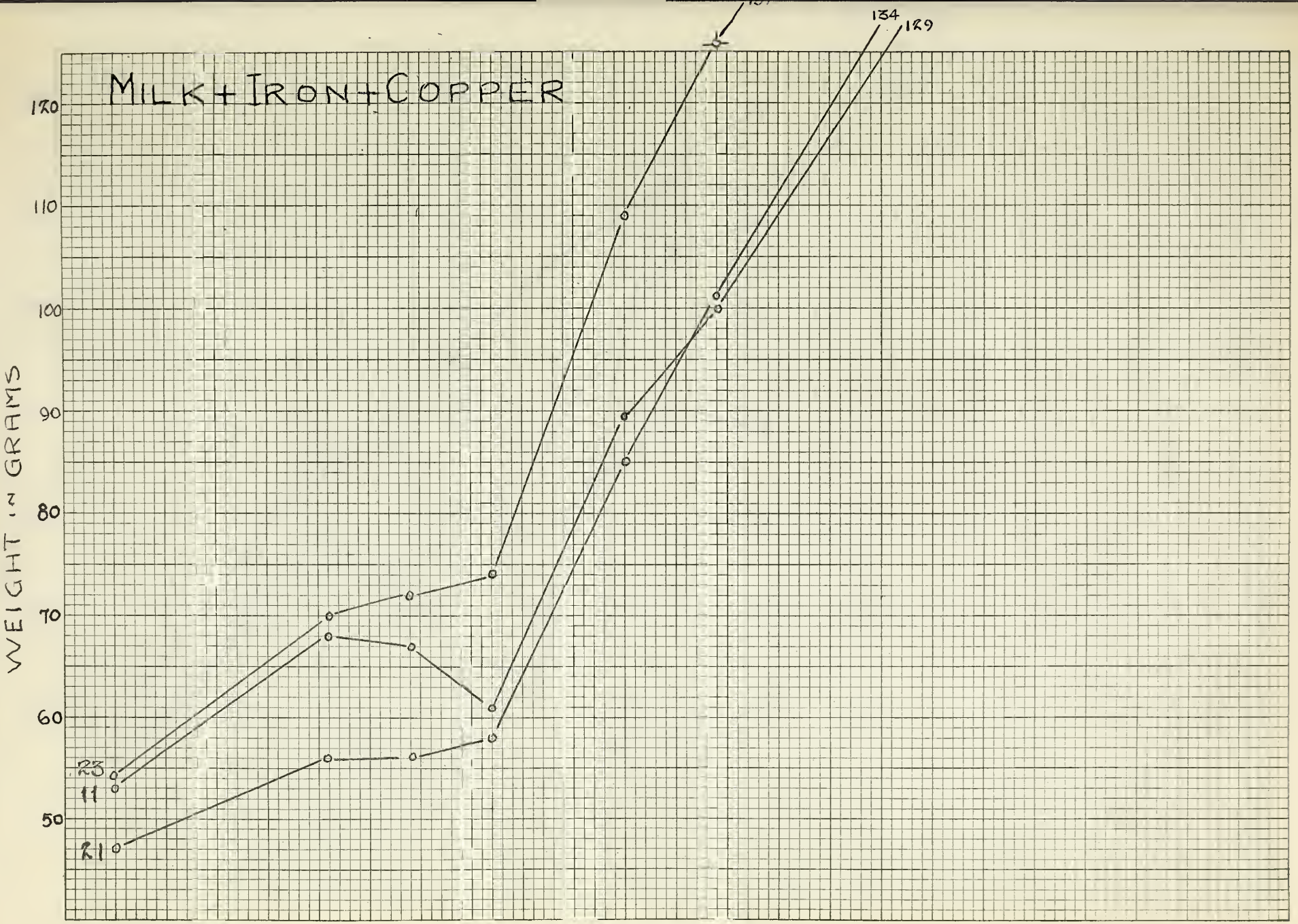

MILK + IRON + COPPER
120
110
100
90
80
70
60
50
WEIGHT IN GRAMS
134
129
R3
11
R1
TIME IN DAYS

FIG VII

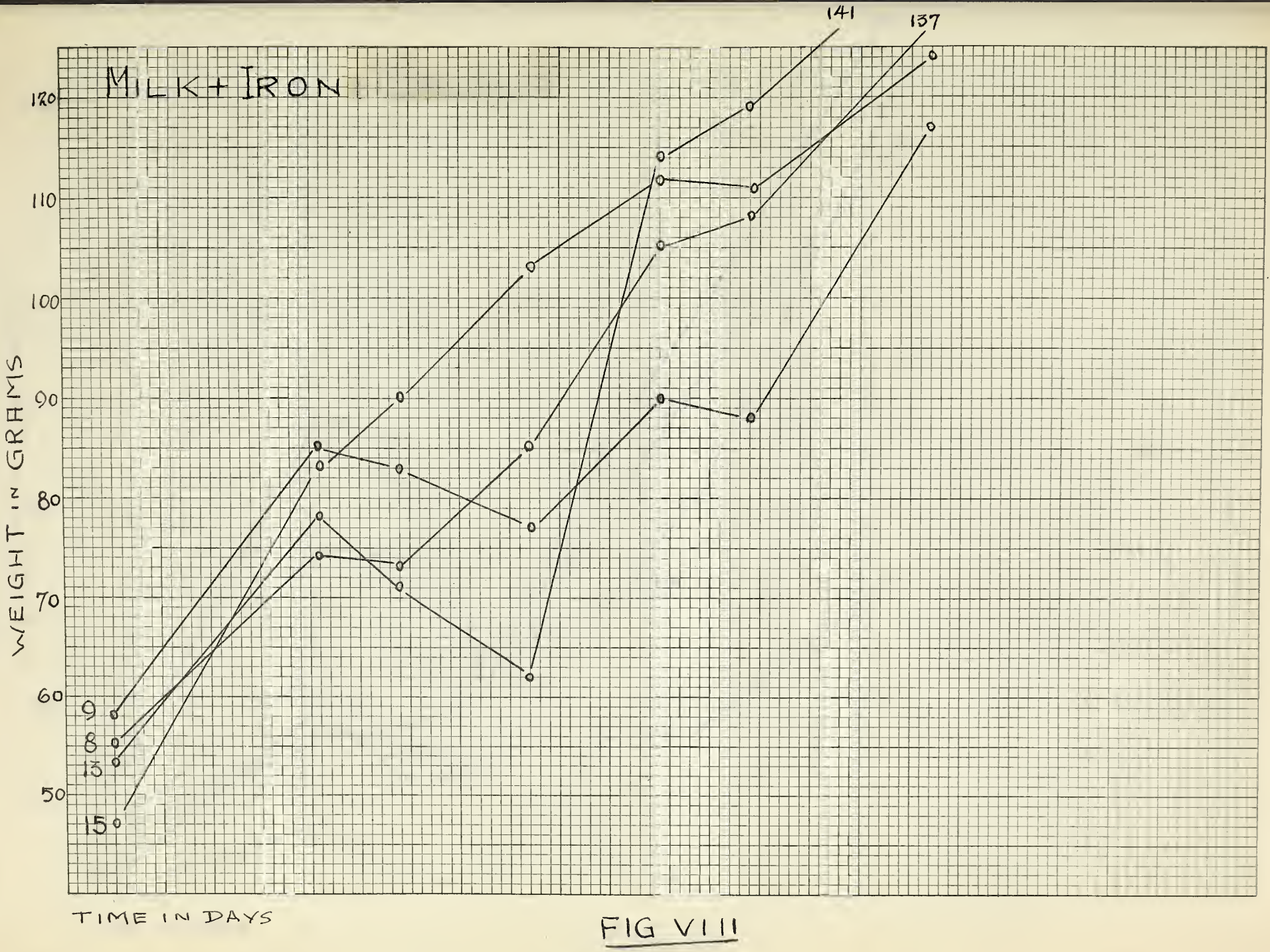

MILK + IRON
141
137
120
110
100
90
80
70
60
50
WEIGHT IN GRAMS
9
8
13
15
TIME IN DAYS

FIG VIII

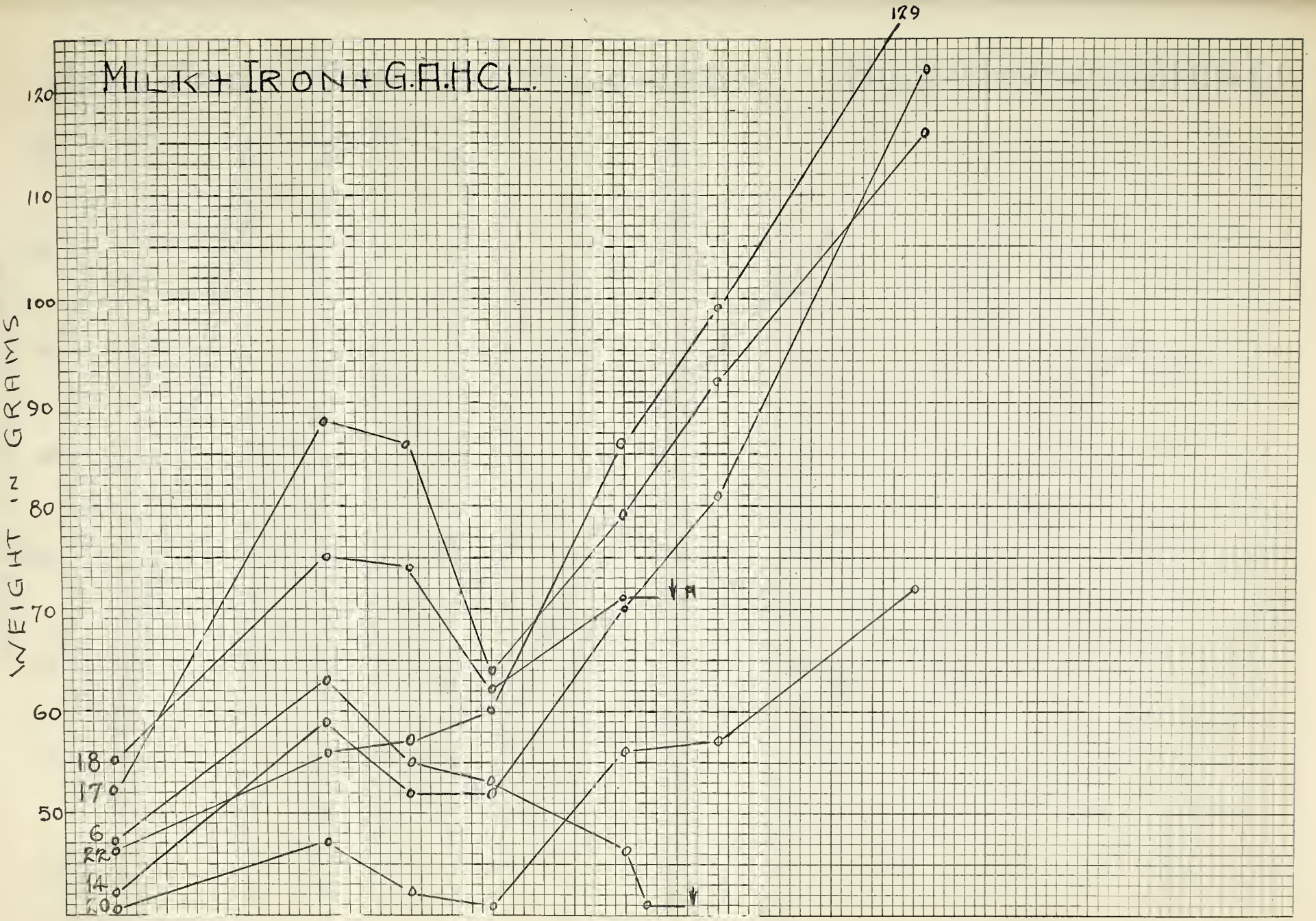

MILK + IRON + G.A.HCL.
129
120
110
100
90
80
70
60
50
WEIGHT IN GRAMS
18
17
6
RR
14
10
TIME IN DAYS

FIG IX

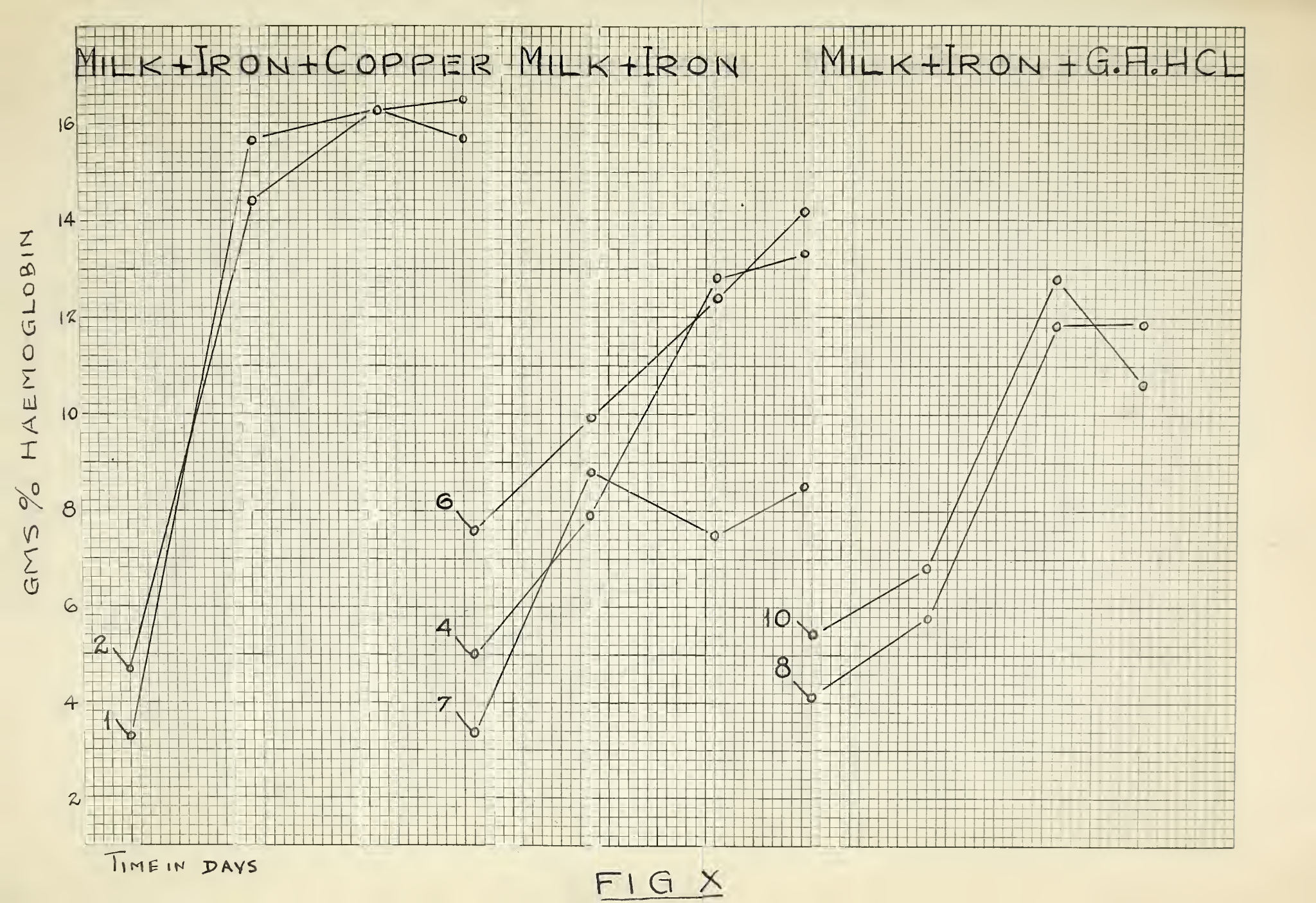

MILK+IRON+COPPER
MILK+IRON
MILK+IRON+G.A.HCL
GMS % HAEMOGLOBIN
16
14
12
10
8
6
4
2
1
2
4
6
7
8
10
TIME IN DAYS

FIG X

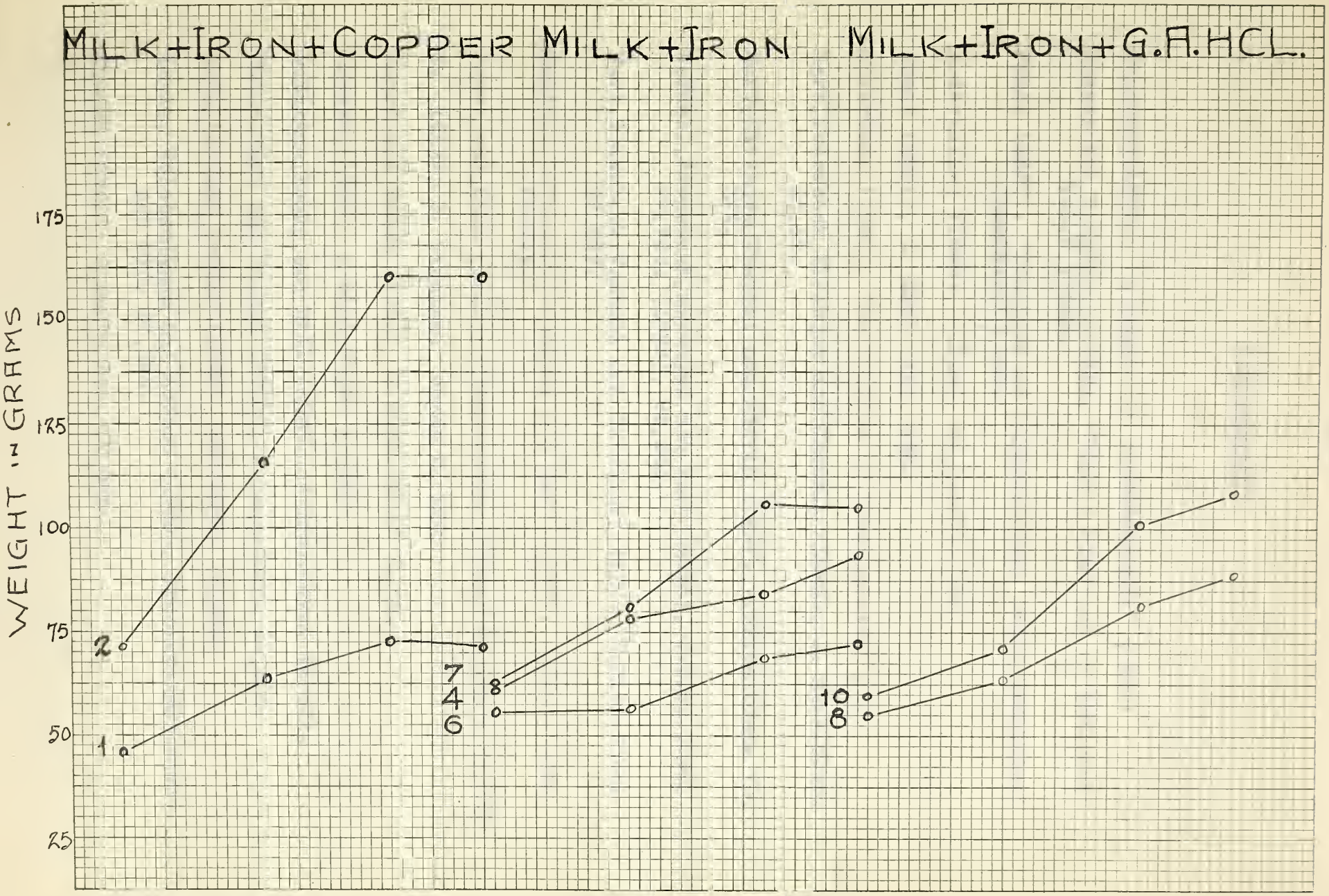

MILK+IRON+COPPER MILK+IRON MILK+IRON+G.A.HCL.
WEIGHT IN GRAMS
175
150
125
100
75
50
25
2
1
7
4
6
8
10
8
TIME IN DAYS

FIG XI

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APPENDIX I

ON THE FACTORS INFLUENCING THE BLOOD-BENZIDINE REACTION AS APPLIED TO THE MICRO ESTIMATION OF HAEMOGLOBIN

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We have been surprised to observe that, notwithstanding the increasing need for an accurate method of determining minute amounts of haemoglobin, the method recommended by Wu, (1923), has not been more generally adopted. Wu developed from the benzidine test for blood a quantitative "Ultra-micro-method" by means of which the haemoglobin content of 0.0001 c.c. of blood could be accurately determined.

There are a number of possible explanations which might account for the lack of favour with which this method has been received, in spite of the unusual merits which it undoubtedly possesses. The complexity of the reaction, or reactions involved, at once raises the question as to whether the many factors which affect the colour development can be properly controlled.

The accuracy of the method proposed by Wu has recently been questioned by Bing and Baker (1931). They have proposed a modified procedure which, they claim, eliminates certain errors in the original method. We have recorded in this paper a critical study of the blood-benzidine reaction, particularly as affected by hydrogen peroxide concentration and temperature. Other observations having a direct bearing on the quantitative application of the reaction are discussed.

EXPERIMENTAL PROCEDURE AND TECHNIQUE

The chemical activity of benzidine, particularly so far as oxidation is concerned, depends on its purity and on the method of preparation. To interpret our experimental results it was essential that benzidine of known purity was employed.

PURIFICATION OF BENZIDINE: To 100 gm. finely powdered commercial benzidine in a two-litre flask were added 900 c.c. boiling water and 100 c.c. concentrated hydrochloric acid. After boiling a short time the hot solution was filtered, using suction. The filtrate was again heated to boiling and 230 c.c. concentrated hydrochloric acid, in 30 to 50 c.c. portions, were added, shaking after each addition until the grey precipitate dissolved. A permanent precipitate forms when about 150 to 175 c.c. have been added. After filtering (suction) the highly coloured filtrate was discarded and the precipitate transferred to a two-litre flask. One litre of boiling water and a tablespoonful of blood charcoal (acid extracted) was added, and after boiling about 10 minutes the hot solution was again filtered (suction). This procedure was repeated until the filtrate was colourless. To the hot filtrate 230 c.c. concentrated hydrochloric acid in 30 to 50 c.c. portions were added while shaking, and after cooling to 20° C. again filtered.

To obtain pure benzidine, 100 gm. of the hydrochloride were suspended in 600 c.c. acetone, 100 c.c. concentrated ammonium hydroxide and 150 c.c. of water. After the benzidine was completely dissolved, the solution was diluted to 5 litres with water and filtered after

standing 20 minutes. The benzidine was washed free from chloride with water, and finally dried over sulphuric acid in a vacuum desiccator. The benzidine thus prepared was found to be extremely insoluble in glacial acetic acid. This difficulty was overcome by recrystallizing from hot 95 per cent. ethyl alcohol.

The benzidine solution used in all our experiments was prepared by dissolving 2 gm. of the pure benzidine in 20 c.c. of glacial acetic acid with the aid of heat, and after cooling, diluting to 100 c.c.

HYDROGEN PEROXIDE: All solutions of hydrogen peroxide were prepared by suitable dilution of "Superoxol". The hydrogen peroxide content of these solutions was carefully checked by permanganate titration, using pure anhydrous sodium oxalate as the primary standard. It was not considered advisable, at least so far as our experiments were concerned, to use the commercial 3 per cent. H_2O_2 solution, which is generally stabilized by acetanilide or by small amounts of sulphuric acid. Hydrogen peroxide forms compounds with sulphuric acid in which the peroxide is less easily decomposable than when alone.

STANDARD BLOOD SOLUTION: The haemoglobin content of a sample of sheep's blood was estimated from its oxygen capacity, as determined by Sendroy's (1931) modification of the Van Slyke and Neill (1924) procedure. This blood was diluted to contain 0.05 mg. haemoglobin in 1 c.c. Boric acid was used as the preservative.

GENERAL EXPERIMENTAL CONDITIONS: Throughout the experiments, special precautions were taken in regard to the cleanliness of all glassware, (see Harrison (1930)). In carrying out the benzidine reaction the reagents were always added in the order recommended by Wu. To 2 c.c.

of a 2 per cent. benzidine solution is added 1 c.c. diluted blood. After carefully mixing, 1 c.c. of a hydrogen peroxide solution is added. After a definite reaction time, the solution is diluted with 21 c.c. 20 per cent. acetic acid, and the intensity of the reddish-brown colour measured in a colorimeter of the Duboscq type.

THE EFFECT OF THE CONCENTRATION OF HYDROGEN PEROXIDE ON THE BENZIDINE REACTION AND ON THE STABILITY OF THE RED COLOUR: The essential feature of the modified procedure recommended by Bing and Baker is the substitution of a much more dilute (0.6 per cent) H_2O_2 solution for the 3 per cent. H_2O_2 solution used by Wu. This permits an extension of the reaction time of 15 minutes, as specified by Wu, to one of one hour or longer, which is an undoubted convenience when many determinations are being made. In their paper Bing and Baker write, "It was found that concentrations of 0.5, 0.6 and 0.75 per cent. (H_2O_2) gave similar good results, while 1.0 and 2.0 per cent. solutions had no apparent advantage". It is particularly unfortunate that the experimental data supporting this contention is not given, since these observations constitute the principal justification for their introducing a modified procedure. We are at a loss to interpret the statement, "gave similar good results". Since they record that 0.6 per cent. H_2O_2 in one hour gave the maximum* colour,

*what they imply by maximum is not defined

we have inferred from the above statement that 1 per cent. and 2 per cent. H_2O_2 with a reaction time of the same duration did not increase the colour intensity.

Our results on the effect of hydrogen peroxide concentration on the development and on the stability of the red colour are given in Table I. The data were obtained as follows: The benzidine reaction was carried out with 1 c.c. of hydrogen peroxide solutions of varying strength. The reaction was allowed to proceed exactly one hour (in all cases) when the solutions were diluted with 21 c.c. 20 per cent. acetic acid. After fifteen minutes the comparative depth of colour was measured, with solution No. 4 (1.5 per cent.H_2O_2) as the arbitrary standard. The test solutions were always set at 10 mm. and the standard adjusted to match. In this way the numerical value of the readings is shown as increasing with increasing intensity of colour. Using a new standard prepared exactly as solution No. 4, the relative stability of the colours was determined after 200 hours. The two standards when read against each other had practically the same depth of colour.

We can conclude that as the concentration of hydrogen peroxide is increased from 0.25 per cent. to 4 per cent.,the intensity of the red

colour also continues to increase. The maximum intensity of the colour consistent with the greatest stability was obtained with 1.5 per cent. or 2 per cent. H_2O_2. 0.75 per cent. H_2O_2 gave only 76 per cent. of the colour given by 1.5 per cent. H_2O_2.

In Table II we have recorded the results obtained when a wider range of H_2O_2 concentrations was employed. The effect of the peroxide concentration on the stability of the red colour was determined hourly up to 4 hours. Again 1.5 per cent. H_2O_2 was the lowest concentration which gave the maximum depth of colour, stable for at least 4 hours. Hydrogen peroxide concentrations of 4 per cent. or greater gave more colour, but the colour was less stable, the instability increasing with increasing H_2O_2 concentration. The intensity of the colour given by 1 per cent. and 0.5 per cent. H_2O_2, which is initially much less than that given by 1.5 per cent. H_2O_2, slowly increased when the solutions were allowed to stand 4 hours.

The results of a further experiment similar to those already described, but in which the reaction time was varied, are given in Table III. By "reaction time " is implied, the length of time the benzidine reaction is allowed to proceed before dilution to 25 c.c. with 20 per cent.

acetic acid. The conclusion may be drawn from these results that 1.5 per cent. H_2O_2 and a reaction time of 45 to 60 minutes gave the maximum depth of colour, the intensity of which undergoes little or no change on standing 15 hours. The stability of the colour with 3 per cent. H_2O_2 when the reaction time is 15 to 45 minutes is noteworthy.

Consideration of the data obtained from these experiments suggests that the benzidine reaction may be carried out with the greatest sensitivity, accuracy and convenience, when 1.5 per cent. H_2O_2 with a reaction time of one hour is employed.

THE EFFECT OF TEMPERATURE ON THE COLOUR DEVELOPMENT: To study the effect of temperature, the following procedure was adopted: The benzidine reactions were carried out in 25 c.c. glass-stoppered measuring cylinders. These were placed in a constant temperature water bath. From micro burettes were then added 2 c.c. 2 per cent.benzidine solution and 1 c.c. diluted blood (0.05 mg. haemoglobin) and the solutions thoroughly mixed. They were then allowed to stand until the reagents came to the temperature of the water bath. This was found to take about three minutes. One c.c. of 1.5 per cent.H_2O_2 was then added. At the end

of the particular reaction time the cylinder was removed from the water bath and 21 c.c. of ice-cold (about 2° C.) 20 per cent. acetic acid was quickly added. The colorimeter readings were made fifteen minutes later.

The benzidine reaction was also carried out in the same manner at 36° F. (refrigerator room).

The temperature at which the benzidine reaction is performed has evidently a marked effect upon the rate of the "initial" stage of the reaction through the blue colour to the red. When the reaction was carried out at 36° F. the solution was still deep blue in colour after a reaction time of 1 hour. At 50° C. a reaction time of 2.5 minutes gave the same depth of colour as that obtained in 15 minutes at room temperatures. It would appear that after the red colour has developed variations in temperature from 20° C. to at least 40° C. have very little effect on the rate at which this colour reaches its maximum intensity . The depth of colour developed after 60 minutes, using 1.5 per cent. H_2O_2 is the same whether the reaction is carried out at 20° C. or at 40° C.

The results of a further experiment in which the reaction was carried out with three different strengths of hydrogen peroxide, and in which the temperature was carefully controlled, are shown graphically in Fig. 1. When the temperature is constant at 30° C., increasing the concentration of H_2O_2 not only accelerates the "initial" stage of the reaction, but the time required for the red colour to reach an intensity equal to that of the standard is markedly shortened.

THE EFFECT OF LIGHT ON THE COLOUR DEVELOPMENT: When the benzidine reaction, using 1 per cent. H_2O_2, was carried out in the dark and in the light we found no difference in the depth of colour developed after a reaction time of one hour.

A few experiments were carried out to determine whether the rate of the reaction was influenced by emanations in the ultra-violet. It was only possible to draw very general conclusions from these results, because of the difficulty in controlling other experimental conditions. Bernheim and Dixon (1928) have found that in studying the effect of light on the reduction of methylene blue by the xanthine oxidase system with oxygen present, a peroxide is formed during the irradiation. There was no doubt, however, that exposure of the reaction mixture to the

radiations of a quartz mercury-vapour lamp, set 12 inches directly above the solution, greatly increased the rate of the reaction. An exposure of 6 to 7 minutes gave about the same depth of colour as ordinarily develops in one hour in daylight. A cloudiness developed in the reaction mixture if irradiated for more than 7 minutes.

THE EFFECT OF VARYING HAEMOGLOBIN CONCENTRATIONS ON THE INTENSITY OF THE COLOUR: Varying amounts from 2 c.c. to 20 c.c. of a dilute blood solution containing 13.5 mgs. of haemoglobin in 100 c.c. were measured into 25 c.c. volumetric flasks. After diluting to volume, the benzidine reaction (2 c.c. benzidine solution; 1 c.c. 1.5 per cent.H_2O_2; room temperature and reaction time 1 hour) was carried out with 1 c.c. aliquots of the different blood dilutions. 21 c.c. of 20 per cent. acetic acid were then added and the solution allowed to stand 10 minutes before making the colorimeter readings. The range of haemoglobin concentration was from 0.01 mg. to 0.10 mg. The fifth dilution (10 c.c. of diluted blood in 25 c.c.) was taken as the arbitrary standard.

In Fig.2 is plotted the colorimeter readings of this standard when adjusted to match each of the other test dilutions which were set at 10 mm. The depth of colour is directly proportional to the amount of haemo-

globin present, provided the standard selected is used over a limited range of haemoglobin concentrations.

THE DETERMINATION OF HAEMOGLOBIN IN SMALL AMOUNTS OF BLOOD: For some considerable time we have used Wu's benzidine method for the routine estimation of haemoglobin in minute amounts of rat blood. As a result of the experimental findings already discussed, certain changes have been made in the original procedure. These modifications constitute an improvement only so far as technique is concerned.

The accuracy with which the haemoglobin content of rat blood can be repeatedly determined is limited primarily by the error incurred in measuring small samples of blood from a free flowing drop taken from the tail. We have used a red cell counting pipette which was accurately calibrated and found to deliver 0.00245 c.c. blood from the 0.05 mark. The accuracy with which samples of blood could be procured by means of this pipette was determined as follows:

The tail was wiped clean (if the rat were anaemic the tail was immersed for a time in warm water) and the end clipped off. The sample was pipetted from the second or third drop of blood, and transferred to a 10 c.c. volumetric flask containing a small amount of water with which

to flush the pipette. The blood solution was diluted to 10 c.c., thoroughly mixed, and the benzidine reaction carried out in the same manner as in conducting the proportionality tests. A number of samples were taken from the same and from different rats. The final colorimeter readings were all made against the same standard (0.028 mg. haemoglobin) which was adjusted to match the test solution set at 10 mm.

In all the experiments described, specially purified benzidine has been used. Quantitative determinations of equal accuracy may be made, using Merck's Benzidine "Suitable for detecting blood".

	Sample of Blood	Colorimeter Reading		Sample of Blood	Colorimeter Reading
Rat No. 1	1	12.0	Rat No. 2	1	9.8
	2	11.5		2	9.4
	3	11.5		3	9.4
	4	11.7		4	10.0
Rat No. 3	1	9.8	Rat No. 4	1	13.0
	2	9.8		2	12.8
	3	10.2	Rat No. 5	1	12.7
	4	10.0		2	12.2

The maximum variation in these colorimeter readings is 0.6 mm., which may be attributed almost entirely to inaccuracies in the measurement of small samples of blood by the method described. The

maximum error of 0.6 mm. is equivalent to an error of 0.7 gm. per cent. haemoglobin in the original sample of blood.

So far we have been primarily concerned with the empirical standardization of the benzidine reaction, so that it might be applied to comparative haemoglobin determinations in rat's blood. Haemoglobin estimations on the same sample of oxalated blood have been made by the colorimetric method and by oxygen capacity determinations. Since the colorimetric method is based on the oxygen capacity determinations, a comparison of the results obtained by the two methods is of no significance, so far as the absolute accuracy of the colorimetric method is concerned. However, since new standards have to be prepared from time to time, the relation of one series of colorimetric determinations to another depends upon the accuracy with which the oxygen capacity determinations check with the colorimetric method.

	Sample of Blood	Grams Haemoglobin in 100 c.c. Blood Colorimetric Method	Calc. from O_2 Capacity Sendroy, 1931)	
Human Blood	*(1	14.3		
	(2	13.8	13.93)	Average 14.08
	(3	13.7	14.23)	
	(4	13.7		

*Aliquots of the same sample of blood.

Sheep Blood	1	13.9	13.97
	2	13.6	
	3	14.2	
Rat Blood No. 3	1	14.0	14.4
	2	14.0	
Rat Blood No. 4	1	10.1	9.64
	2	9.9	

THE EFFECT OF HEATING THE DILUTED BLOOD: Three 10 cc.aliquots of a diluted blood (13.5 mg. haemoglobin in 100 cc.) were measured into 25 cc. volumetric flasks. Two of the flasks were placed in a constant temperature water bath at 60° C. One was removed at the end of thirty minutes, and cooled to room temperature, while the other remained in the water bath for one hour. The flasks were all diluted to 25 cc. then thoroughly mixed and the benzidine reaction carried out on a 1 cc. aliquot (0.054 mg. haemoglobin).

	Treatment of Diluted Blood	Colorimeter Readings in m.m. of No.1 as Standard when other test solutions set at 10 mm.
No. 1	Unheated	10.0
No. 2	Heated for 30 min. at 60° C.	9.1
No. 3	Heated for 1 hour at 60° C.	9 .0

These observations have been repeated independently by both

authors with samples of blood from different sources in human, sheep and rat blood. About 10 per cent. of the peroxidase activity is destroyed by heating the diluted blood at 60° C. for 30 minutes. The activity is unchanged when the sample is held at the same temperature for one hour.

GENERAL DISCUSSION

To those familiar with the blood-benzidine reaction, it is evident that the quantitative application is only possible when the use of each reagent has been standardized. In this way only can those conditions be maintained which are necessary to preserve a constant peroxidase activity of the blood. The amount of benzidine transformed into the red oxidation product in a given time should then be directly proportional to the quantity of peroxidase (haemoglobin) present.

The greatest difficulty encountered in controlling the reaction so that it may form the basis of a colorimetric procedure which is free from objections, is to establish the optimum concentration of hydrogen peroxide. According to Wu, when 3 per cent. H_2O_2 is used, the peroxidase reaction reaches a maximum in about 25 minutes, the "haemoglobin being all destroyed by the peroxide". The depth of colour, after 25 minutes, slowly increased, "due to direct oxidation of the benzidine by the

peroxide alone". No explanation is offered as to how the cessation of peroxide activity (indirect oxidation) was differentiated from the commencement of the direct oxidation of the benzidine by the hydrogen peroxide. Even more indefinite and confusing is the statement of Bing and Baker that "We feel these better results (using 0.6 per cent. H_2O_2) are due in part to a lessened tendency for the destruction of the haemoglobin activity by stronger peroxide solutions, and in part to the avoidance of the direct oxidation of the benzidine".

It is at once obvious that if these observations are tenable they preclude any possibilityof developing an accurate quantitative colorimetric method based on the benzidine blood reaction. We can find no evidence to support the supposition that the benzidine may be directly oxidized by the excess hydrogen peroxide, or that the peroxidase activity of oxyhaemoglobin may be destroyed by stronger (presumably 3 per cent.) peroxide solutions when benzidine is also present. The destruction of the peroxidase activity of dilute blood by hydrogen peroxide in the absence of benzidine is no evidence that the same reaction may take place in the presence of a readily oxidized substance. On the contrary, Willstatter and Stoll (1918) have shown that the catalytic function of

oxyhaemoglobin, in contrast to the true plant peroxidase, increases pronouncedly with rising hydrogen peroxide concentration. Oxyhaemoglobin acts as a peroxidase without accelerating the destruction of hydrogen peroxide to any detectable extent (see Kuhn and Brann, (1926)). Again, our own observations on the increase in colour intensity with increasing concentrations of hydrogen peroxide from 0.5 per cent. to 10 per cent. (see Table II), while difficult to interpret, certainly do not support the above contentions. For the sake of clarity the first series of readings recorded in Table II are plotted in Fig. 3.

A mixture of 2 cc. of our benzidine solution and 1 cc. 3 per cent. H_2O_2, when allowed to stand at room temperatures for two hours, developed only a faint yellow tinge. There is no reason to suspect that direct oxidation of the benzidine by 3 per cent. H_2O_2 is any more likely to occur when the dilute blood is present. The values plotted in Fig. 3 represent the amounts of substrate (benzidine) oxidized by varying amounts of H_2O_2 in a given time (1 hour) with a constant quantity of the catalyst present. The amount of substrate oxidized in one hour increases with increasing H_2O_2 concentrations, and reaches a maximum with 8 per cent. H_2O_2.

To form the basis of an accurate colorimetric method, the optimum concentration of H_2O_2 is considered to be that which gives the maximum depth of colour consistent with greatest stability. Under the conditions of our experiments this was found to be 1.5 per cent. hydrogen peroxide.

The literature contains ample evidence to support the conclusions that the leucocytes contain a true (thermolabile) peroxidase. At such large dilutions as those employed above, one would conclude the true peroxidase activity to be quantitatively negligible compared with the pseudo peroxidase. (thermostable) activity of oxyhaemoglobin. Ten per cent. of the peroxidase activity of diluted blood is destroyed by heating at 60° C. for 30 minutes. Whether this is due to the destruction of a thermolabile peroxidase or to some change in the oxyhaemoglobin molecule, remains to be determined. Kuhn and Brann (1926) have shown that relatively unimportant chemical alterations in the haemoglobin molecule do markedly change the peroxidase activity. When the opportunity presents itself we plan to repeat our observation on the effect of heating the diluted blood, using pathological bloods with a very high leucocyte content, such as in leukaemias. Delhougne

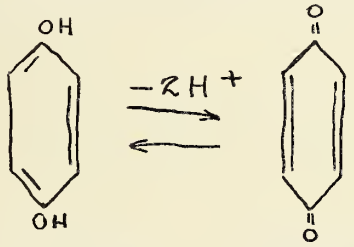
(1929) has reported that the true peroxidase number of blood as determined by the method of Bach and Zubkowa (1921) is sharply increased by vigorous exercise and in pathological conditions.

The chemistry of the blue and finally red oxidation products of benzidine is not well understood. The blue intermediate oxidation product is extremely unstable and is rapidly oxidized to the final red product. It is well known that these partial oxidation products of benzidine resemble the highly coloured meriquinones of Willstatter and Piccard (1908). These meriquinones represent intermediate stages in the reduction of quinones to hydroquinone. The colour of their solution depends on the ratio of the reduced to the fully oxidized (holoquinone) forms.

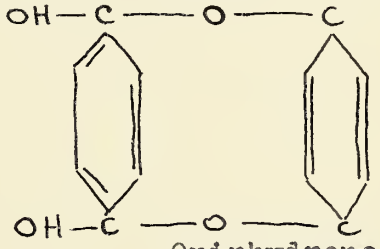
Clark, Cohen and Gibbs (1926) were unable to obtain well defined potentials in redox systems involving the meriquinones of the benzidine series. The instability of these systems has prompted them to warn against the drawing of conclusions, particularly of a quantitative nature, from colour reactions with benzidine. Aside from the fact that these electrode measurements, made in aqueous buffer solutions, can only apply to the reaction under consideration to a very limited

extent, we were inclined to disregard them entirely on the grounds that the blood-benzidine reaction is irreversible. While we have been unable to find any definite evidence to the contrary, Michaelis (1930) has recorded that a powerful intra-molecular redox potential is exerted by the oxidized and reduced products of benzidine half combined together in the same molecule.

When the blood-benzidine reaction is carried out under the controlled conditions which we have described, the final red oxidation product is extremely stable, even when the reaction is carried out at 50°C. While the nature of the tie between quinone and hydroquinone in quinhydrone is not definitely known, according to Porter (1924) a structure representing quinhydrone as an addition compound has been proposed.

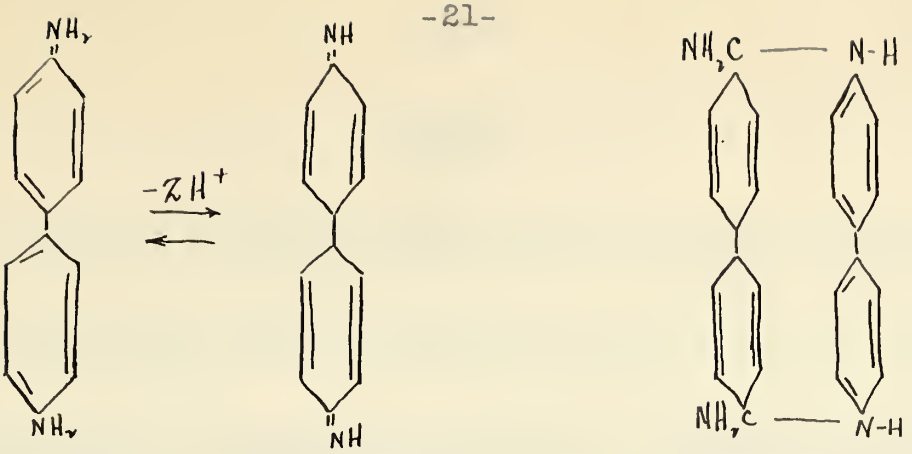


Hydroquinone Quinone



Quinhydrone

The stability which we have found the red oxidation product of benzidine to possess can perhaps be best explained by postulating a similar type of linkage between equimolecular concentrations of the oxidized and reduced forms.



Benzidine Holoquinone Meriquinone

The blue coloured oxidation product which is extremely unstable, probably represents a meriquinone in which the ratio of the oxidant to the reductant is variable, such as in Wurster's blue.

SUMMARY

I. Certain factors influencing the blood-benzidine reaction have been studied, with the following results:

a. The amount of benzidine oxidized in one hour by hydrogen peroxide in the presence of a fixed amount of the catalyst (haemoglobin) increases with increasing hydrogen peroxide concentration. A maximum is reached with an 8 per cent. solution of hydrogen peroxide. The maximum amount of the red oxidation product which is stable for at least twelve hours, under the conditions of the blood-benzidine reaction, is given by a 1.5 per cent. solution of hydrogen peroxide.

b. The "initial" reaction, through the blue colour to the red, is markedly accelerated by increasing temperature. The rate at which the red colour reaches its maximum intensity is not influenced by variations in temperature from 20° C. to 40° C.

c. Light has no effect on the colour development. Radiations from a quartz mercury-vapour lamp greatly increase the rate of the reaction.

d. Heating diluted blood at 60° C. for 30 minutes destroys about 10 per cent. of its peroxidase activity. The inactivation is not increased by heating for one hour at 60° C.

e. When the reaction is carried out, using a 1.5 per cent. solution of hydrogen peroxide, and a reaction time of one hour at room temperatures, the peroxidase activity within the range of dilutions studied, is proportional to the concentration of haemoglobin.

II. The benzidine reaction, modified in accordance with the results summarised above, has been applied to the estimation of haemoglobin in minute amounts of blood. The accuracy of these estimations depends primarily upon the accuracy with which small samples (e.g. 0.0025 cc.) can be measured.

III. Some theoretical considerations of the factors influencing the blood-benzidine reaction are submitted.

TABLE I

THE EFFECT OF H_2O_2 CONCENTRATIONS ON THE BENZIDINE REACTION

Reaction Time, 1 hour Temperature 20-22° C.

Solution Number	Percentage Concentration of H_2O_2	Reading of the Standard (No.4) in mm. when the other test solutions set at 10 mm.	
		After 15 minutes	After 22 hours
1	4.0	11.8	9.8
2	3.0	11.1	9.8
3	2.0	9.9	10.3
4	1.5	10.0	10.0
5	1.0	8.4	10.3
6	0.75	7.6	10.2
7	0.50	6.3	8.8
8	0.25	5.1	7.9

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5	1.0	8.4	10.3
6	0.75	7.6	10.2
7	0.50	6.3	8.8
8	0.25	5.1	7.9

EFFECT OF H_2O_2 CONCENTRATION ON THE DEVELOPMENT AND STABILITY OF THE RED COLOUR

Reaction Time One Hour, Temperature 20-22° C.

Solution No.	Percentage Concentration H_2O_2	Reading of Standard, when other test solutions set at 10 mm.			
		* 15 Minutes	2 Hours	3 Hours	4 Hours
1	10	11.8 mm	10.5 mm	8.8 mm	7.9 mm
2	8	12.0 "	11.2 "	9.7 "	8.7 "
3	6	11.6 "	10.2 "	9.4 "	8.5 "
4	4	11.2 "	11.0 "	10.5 "	10.0 "
5	2	10.3 "	10.2 "	9.9 "	9.9 "
6	1.5	10.0 "	10.3 "	10.2 "	10.2 "
7	1.0	8.4 "	8.9 "	9.8 "	10.2 "
8	0.5	6.8 "	7.4 "	8.4 "	8.9 "

Tube No. 6 (1.5% H202) was used as standard in the first series of readings, i.e. after 15 minutes. A fresh standard prepared exactly as No. 6 was used for each series of readings.

* Time after dilution with 20% acetic acid.

EFFECT OF VARYING CONCENTRATIONS OF H_2O_2 ON THE DEVELOPMENT AND STABILITY OF THE RED COLOUR

Reaction time varying, room temperatures prevailing

Reading of standard when other test solutions set at 10 mm.

Solution No.	Reaction Time	3% H_2O_2				1.5% H_2O_2				0.5% H_2O_2	
		15 Minutes	2 Hrs.	3 Hrs.	15 Hrs.	15 Min.	2 Hrs.	3 Hrs.	15 Hrs.	15 Minutes	15 Hours
1	2 hours	* -	-	-	-	13.1	12.7	12.8	11.9	9.5	10.6
2	1.5 "	-	-	-	-	11.2	10.9	11.0	9.8	8.7	10.1
3	1.0 "	12.6	12.0	11.6	10.3	10.0	10.3	10.3	9.8	8.1	10.0
4	45 min.	11.3	11.0	11.1	9.3	10.1	10.0	10.2	10.0	7.6	9.3
5	30 "	10.8	10.7	10.9	9.5	9.6	10.1	10.3	9.8	6.7	9.0
6	15 "	10.2	10.0	10.0	8.4	8.1	8.9	9.1	9.0	5.2	8.8

Note: A freshly prepared standard was used for each series of readings after 15 minutes, two hours etc. The preparation of the standard was always the same, i.e. using 1.5% H202, reaction time 1 hour, and allowing the solution to stand 15 minutes after dilution with 20% acetic acid.

* Solutions were cloudy.

EFFECT OF TEMPERATURE ON THE BENZIDINE REACTION

Reading of Standard when other test solutions set at 10 mm.

Reaction Time	20-22 °C.	30 °C.	40 °C.	50 °C.	60 °C.
2.5 Minutes	* -	-	-	7.0 mm.	-
3 "	-	-	-	-	-
5 "	-	5.0 mm	7.0 mm	8.0 mm	7.2 mm
10 "	-	6.7 "	-	8.2 "	7.4 "
15 "	7.1 mm	7.1 "	8.2 "	8.6 "	7.5 "
20 "	8.3 "	7.8 "	-	8.6 "	-
25 "	8.8 "	8.5 "	-	9.4 "	-
30 "	9.2 "	8.5 "	9.2 "	9.4 "	7.5 "
45 "	10.2 "	9.5 "	9.7 "	9.6 "	Cloudy
60 "	10.0 "	10.0 "	9.9 "	9.2 "	-

These experiments werecompleted on the same day, so that the same standard, using 1.5% H202 and a one-hour reaction time at room temperatures, was used for all readings. 1.5% H202 was used in all the tests.

* The reaction had not proceeded to the red colour. The solution after dilution with 20% acetic acid had a greenish-red colour.

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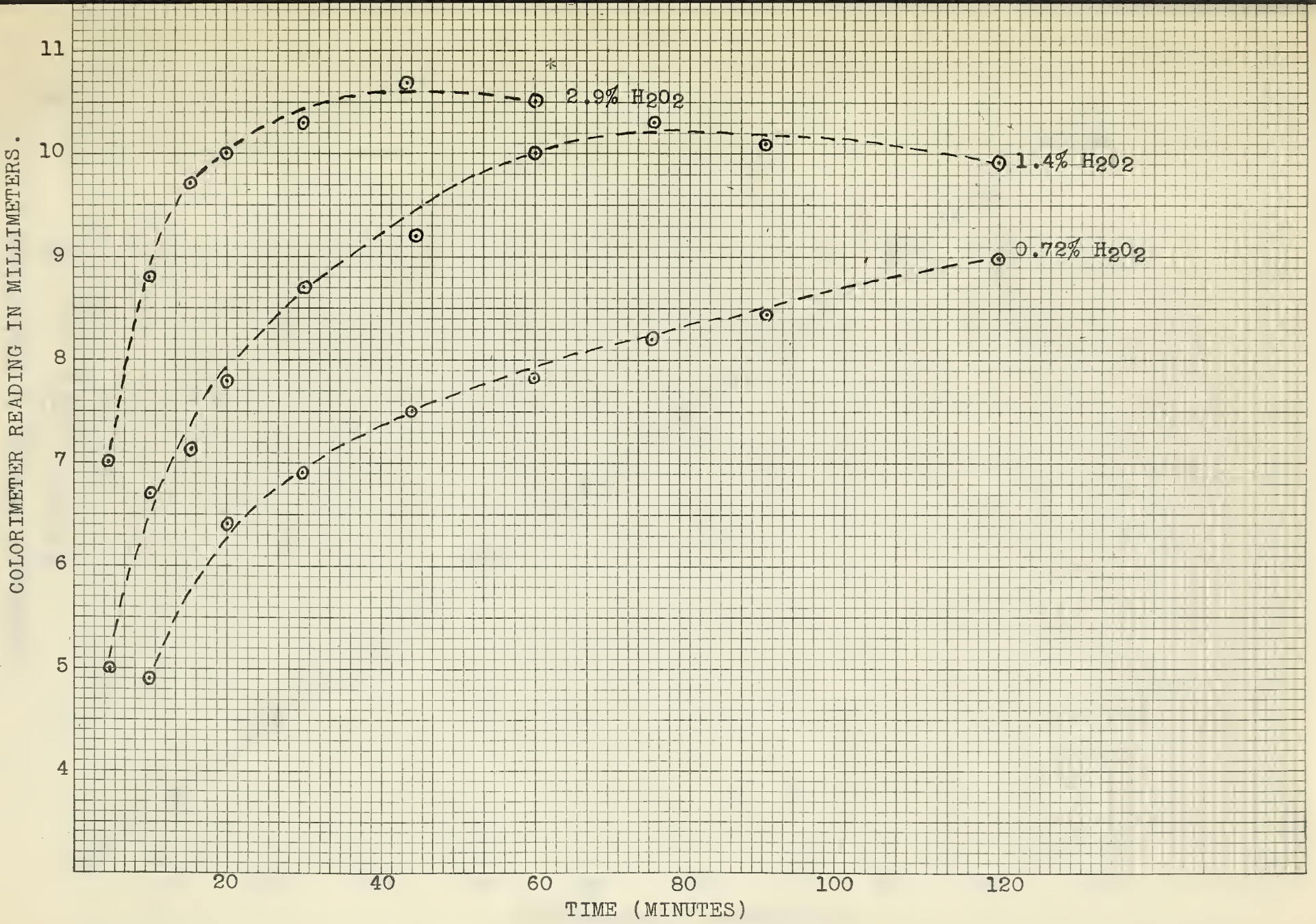


Fig. 1. Showing the effect of varying concentrations of H_2O_2 on the rate of substrate (benzidine) oxidation. * Solutions became cloudy.

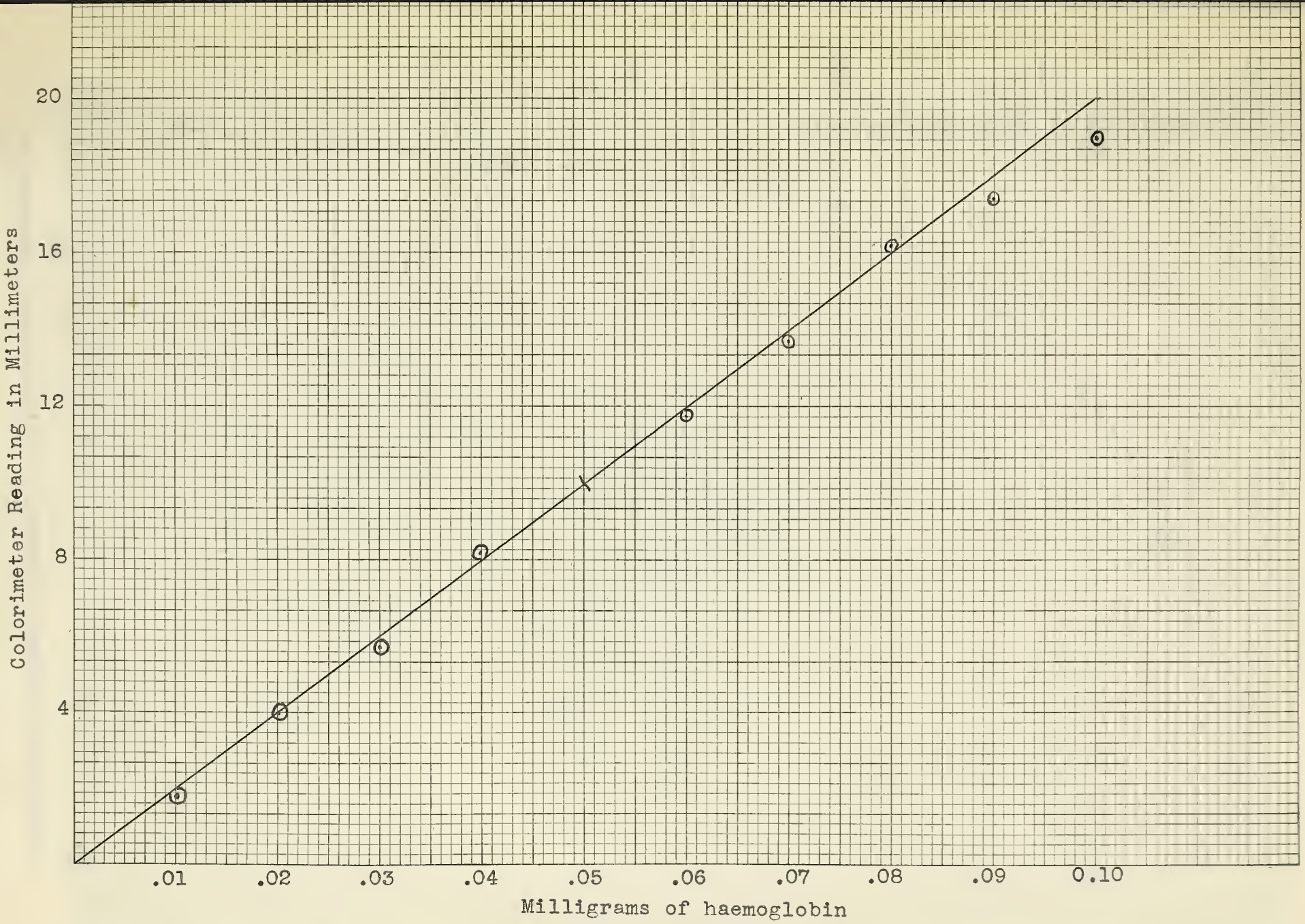
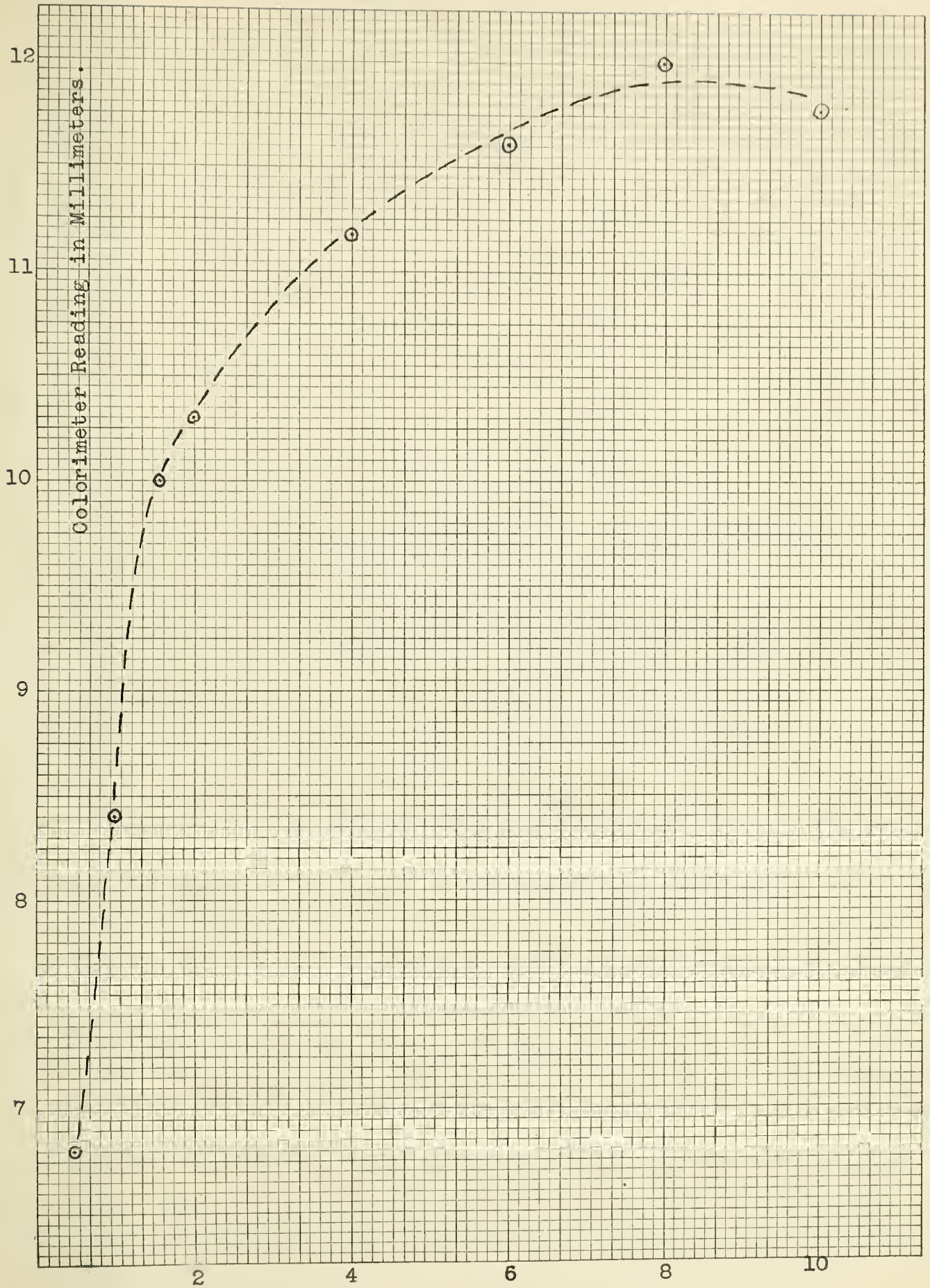


Fig. 2. Showing the relationship between the haemoglobin concentration and the intensity of the red colour.

Fig. 3.


Colorimeter Reading in Millimeters.
12
11
10
9
8
7
2
4
6
8
10
Percentage concentration of hydrogen peroxide solutions.

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